THE UNIVERSITY OF ALBERTA

REGULATION OF CHLORIDE CONDUCTANCE IN CULTURED EPITHELIAL CELLS

by

MELISA WAI YIN HO ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirement for the degree of **Doctor of Philosophy**.

DEPARTMENT OF PHYSIOLOGY

EDMONTON ALBERTA

SPRING 1997

National Library of Canada

Acquisitions and Bibliographic Services

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada

Acquisitions et services bibliographiques

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced with the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-21577-6

Canadä

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: Melisa Wai Yin Ho

TITLE OF THESIS: Regulation of chloride conductance in cultured epithelial cells

DEGREE: Doctor of Philosophy

YEAR THIS DEGREE GRANTED: Spring 1997

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as hereinbefore provided neither the thesis nor substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.

Melisa Wai Yin Ho
University of Alberta
Department of Pharmacology
Edmonton, Alberta
T6G 2H7

Date: 20 Jan '97

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled **REGULATION OF CHLORIDE CONDUCTANCE IN CULTURED EPITHELIAL CELLS** submitted by MELISA WAI YIN HO in partial fulfilment of the requirement for the degree of DOCTOR OF PHILOSOPHY.

A.S. French
Dr. Andrew S. French (Supervisor)

Dr. Marek Duszyk (Co-supervisor)

John Cuppoletti
Dr. John Cuppoletti (External examiner)

Dr. Anthony K. Ho

Dr. Chris I. Cheeseman

Susan M J Dunn
Dr. Susan Dunn

Dr. Susan Jacobs (Chairperson)

Date: 20 Jan '97

To my mother, for everything she has given me

and

To Verne, for his love and support

ABSTRACT

Ion transport is a major function of epithelial tissues. To perform this transport, epithelial cells have several types of transport components, whose locations are crucial to the overall function. A variety of different apical Cl^- channels have been found in secretory epithelia. Opening of these Cl^- channels is controlled by different combinations of regulatory mechanisms. This thesis is concerned with Cl^- channels in two epithelial cell types, and their regulation by cell swelling and internal Ca^{2+} concentration.

A swelling-activated Cl^- conductance in the T84 epithelial cell line was examined using the whole-cell patch clamp technique. Swelling activated an inwardly rectifying Cl^- current, independent of intracellular calcium concentration ($[Ca^{2+}]_i$), and intracellular ATP. Noise analysis was used to estimate the single-channel conductance and number of Cl^- channels responsible for the swelling-activated current. This showed that swelling activates Cl^- channels with conductance below 1 pS. However, the significant current produced by these channels indicates that they are present in relatively large numbers.

The effect of extracellular ATP on epithelial Cl^- permeability was examined in the CFPAC-1 cell line. This regulation seems to operate via changes in $[Ca^{2+}]_i$. Extracellular ATP produced a biphasic increase in $[Ca^{2+}]_i$ that was not prevented by

removal of extracellular Ca^{2+}. However, depletion of intracellular Ca^{2+} stores with thapsigargin abolished the effect of ATP on $[Ca^{2+}]_i$, indicating that ATP-activated surface purinergic receptors mobilize Ca^{2+} from intracellular stores via an Ins(1,4,5)P_3-dependent mechanism. The ATP-induced increase in $[Ca^{2+}]_i$, in turn, activated an outwardly rectifying whole-cell Cl^- current.

In addition to InsP_3, there is accumulating evidence that other inositol phosphates, generated by activation of phospholipase C-linked receptors, can have regulatory effects on cellular processes. Therefore, the effects of several other products of the inositol phosphate cascade were investigated for regulatory effects on the ATP-mediated, Ca^{2+}-dependent Cl^- current. Ins(3,4,5,6)P_4 specifically inhibited the Ca^{2+}-dependent Cl^- current. Three other InsP_4 isomers, Ins(1,4,5,6)P_4, Ins(1,3,4,5)P_4 and Ins(1,3,4,6)P_4, had no inhibitory effect on the Ca^{2+}-dependent Cl^- current, but instead enhanced the effects of ATP via Ca^{2+}-dependent mechanisms.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisor Dr. Andrew S. French for his guidance in this work. Most important of all, I thank him for being so understanding of my personal decision to remain in Edmonton. Without his patience, and the wonder of E-mail, this long distance relationship would not have been possible. I would also like to thank the Chairman of this department, Dr. Esmond Sanders for his concern and allowing me to continue my studies in this department. Dr. Marek Duszyk provided me with technical advice that I have valued throughout my study. I am also grateful for all the help I received from the following people; Dr. Anthony K. Ho for his advice on signal transduction pathways; Dr. Ed Karpinski for his advice on calcium measurement and his unlimited supply of platinum wire; Dr. Peter Pang for allowing me to use the calcium imaging setup; Dr. Stephen B. Shears for his critical comments on the inositol phosphates study; Mabel Ritzel for her advice on cell culture, and Li Ping for his technical assistance on the calcium measurement experiments.

My work was made possible by studentship awards from the Canadian Cystic Fibrosis Foundation and the Alberta Heritage Foundation for Medical Research. Support for the research was also provided by these two agencies.

I appreciate the friendships that I have made with my colleagues Dan Liu, Ewa Parker, Päivi Torkkeli, Gillian Ridge, Beata Kamosinska, and Mikko Juusola during my study in Edmonton. I thank them for all their encouragement and support in the frustrating days. I would like to thank Sylvia Yao, Mabel Ritzel and Amy Ng for adopting me into their lab and their wonderful companionship during my months of solitude in Room 7-20.

Support from my close friends Singfat Chu, Jason Pao, Alan Brain, Ellen Tsao, and Sonia Venäläinen have made this long and sometimes frustrating journey possible. I would like to express special thank to my good friend Sonia Venäläinen and her family for being there for me all these years.

I would like to express my gratitude to my brother for everything he has done for me. I am also thankful for the generous support and understanding from my aunt Kattie. Finally, without my parents I could not have achieved what I have achieved today.

INDEX

Chapter Page

1. GENERAL INTRODUCTION 1

1.1 Ion transport in secretory epithelia 1
1.2 Cystic fibrosis 4
1.3 Ion channels in epithelia 9
1.3.1 Sodium channels 10
1.3.2 Potassium channels 13
1.3.2.1 Voltage-dependent K^+ channels 13
1.3.2.2 Ca^{2+}-dependent K^+ channels 15
1.3.3 Chloride channels 17
1.3.3.1 cAMP-activated Cl^- channels 17
1.3.3.2 Ca^{2+}-dependent Cl^- channels 20
1.3.3.3 Swelling-activated Cl^- channels 23
1.3.3.4 Other epithelial chloride channels 23
1.4 Cell volume regulation in epithelia 28
1.5 Purinergic receptors 34
1.6 PLC-dependent inositol phosphates cycle 44
1.7 Tissue culture in biological research 56
1.8 Electrophysiological techniques 58
1.9 Outline of the thesis 60

2. MATERIALS AND METHODS 62

2.1 Cell culture 62
2.2 Electrophysiological recording 64
2.3 Intracellular calcium measurements 68
2.4 Noise analysis 70
2.5 Statistical Analysis 72

3. RESULTS 73

3.1 Ionic basis of the swelling-regulated conductance in T84 cells 73
3.1.1 Introduction 73
3.1.2 Experimental data 75
3.2 Characterization of the ion channels responsible for the swelling-activated Cl^- current 80
3.2.1 Introduction 80
3.2.2 Experimental data 81
3.3 Identification of surface purinergic receptors in CFPAC-1 cells 87
3.3.1 Introduction 87
3.3.2 Experimental data 88

3.4 Ionic basis of the Ca^{2+}-dependent current in CFPAC-1 cells 94
    3.4.1 Introduction 94
    3.4.2 Experimental data 95
3.5 Regulation of the Ca^{2+}-dependent Cl^- current by inositol phosphates 103
    3.5.1 Introduction 103
    3.5.2 Experimental data 105

4. DISCUSSION 123

4.1 Cell volume regulation in T84 cells 123
4.2 Surface P_2 purinoceptors in CFPAC-1 cells 129
4.3 Regulation of the purinoceptor-activated, Ca^{2+}-dependent Cl^- current in CFPAC-1 132

5. CONCLUSIONS AND FUTURE DIRECTIONS 140

5.1 Conclusions 140
5.2 Future directions 141

BIBLIOGRAPHY 143

LIST OF TABLES

Chapter 1. **Page**

Table 1.3.1 Molecular properties of the cloned ClC Cl^- channels 26

Table 1.3.2 Electrophysiological properties of the ClC Cl^- channels 27

Chapter 2.

Table 2.2.1 Compositions of the bath solutions 66

Table 2.2.2 Compositions of the pipet solutions 67

LIST OF FIGURES

Page

Chapter 1.

Figure 1.1. General model of ion transport 2

Figure 1.6. The receptor-activated inositol phosphate pathways 50

Chapter 3.1.

Figure 3.1.1. Development of the swelling-activated whole-cell current 76

Figure 3.1.2. Effect of reversed osmotic gradient 78

Figure 3.1.3. Effect of DIDS on the swelling-activated whole-cell current 79

Chapter 3.2.

Figure 3.2.1. Spectral analysis of the swelling-activated whole-cell current 83

Figure 3.2.2. Development of swelling-induced membrane current noise 85

Figure 3.2.3. Calculated open probability versus time 86

Chapter 3.3.

Figure 3.3.1. ATP-induced changes in $[Ca^{2+}]_i$ 89

Figure 3.3.2. Dose-dependent effect of ATP 91

Figure 3.3.3. Effect of TG on ATP-induced changes in $[Ca^{2+}]_i$ 92

Figure 3.3.4. UTP-induced changes in $[Ca^{2+}]_i$ 93

Chapter 3.4.

Figure 3.4.1. ATP-activated whole-cell current 96

Figure 3.4.2. Current-voltage relationships for the ATP-activated whole-cell current 97

Figure 3.4.3. Effect of DIDS on the ATP-activated whole-cell current 99

Figure 3.4.4. Ca^{2+} dependence of the ATP-activated whole-cell current 100

Figure 3.4.5. TG-induced whole-cell current 102

Chapter 3.5.

Figure 3.5.1. Effect of Ins(3,4,5,6)P_4 on the ATP-activated whole-cell current 107
Figure 3.5.2. The mean Ins(3,4,5,6)P_4-inhibited ATP-activated whole-cell current 108
Figure 3.5.3. Dose-dependent inhibitory effect of Ins(3,4,5,6)P_4 109
Figure 3.5.4. Effect of Ins(3,4,5,6)P_4 on the TG-induced whole-cell current 111
Figure 3.5.5. Effect of Ins(1,4,5,6)P_4 on the ATP-activated whole-cell current 112
Figure 3.5.6. Effect of Ins(1,3,4,5)P_4 on the ATP-activated whole-cell current 113
Figure 3.5.7. Effect of Ins(1,3,4,6)P_4 on the ATP-activated whole-cell current 114
Figure 3.5.8. Mean ATP-activated whole-cell currents with different inositol phosphates 115
Figure 3.5.9. Effects of different InsP_4s on whole-cell current without ATP 117
Figure 3.5.10. Ca^{2+}-dependence of Ins(1,3,4,5)P_4 and Ins(1,3,4,6)P_4 effects 120
Figure 3.5.11. Effect of coinjection of Ins(3,4,5,6)P_4 and Ins(1,3,4,5)P_4 or Ins(1,3,4,6)P_4 on the ATP-activated whole-cell current 121
Figure 3.5.12. Effect of Ins(1,3,4,5,6)P_5 on the ATP-activated whole-cell current 122

LIST OF SYMBOLS

Abbreviations

Ins*P*	*myo*-inositol and phosphate substituents in inositol polyphosphates

Acronyms

SITS	4-acetamido-4’-isothiocyanatostilbene-2,2’-disulfonic acid
ADP	adenosine diphosphate
ADPßS	adenosine 5’-*O*-(2-thiodiphosphate)
AMP	adenosine monophosphate
ATPγS	adenosine 5’-*O*-(3-thiotriphosphate)
ATPase	adenosine triphosphatase
ATP	adenosine triphosphate
4-AP	4-aminopyridine
CaMK	calcium/calmodulin-dependent kinase
PKA	cAMP-dependent protein kinase
ChTX	charybdotoxin
cAMP	cyclic adenosine monophosphate
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
DG	1,2-diacylglycerol
DNDS	4,4’-dinitrostibene-2,2’-disulfonic acid
DIDS	4,4’-diisothiocyanostilbene-2,2’-disulfonic acid
DPC	diphenylamine-2-carboxylic acid
DMEM-Ham F12	Dulbecco’s modified Eagle’s medium with Ham’s nutrient mixture F-12
NECA	5’-*N*-ethylcarboxamidoadenosine
EDTA	ethylene-diaminetetra-acetic acid
EGTA	ethylene glycol-bis(ß-aminoethyl ether)-N,N,N’,N’-tetraacetic acid
HEPES	*N*-2-hydroxyethylpiperazine-*N*’-2-ethanesulfonic acid
ID	inner diameter
IMDM	Iscove’s Modified Dulbecco’s medium
AMP-C-PP	*α*,ß-methylene-ATP
2-MeSATP	2-methylthio-ATP
MDR1	multidrug resistance gene
NPPB	5-nitro-2-(3-phenylpropylamino) benzonic acid
OD	outer diameter
ORCC	outwardly rectifying chloride channel
Pgp	P-glycoprotein

S-PIA	(+)-N^6-(phenylisopropyl)adenosine
PLA$_2$	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PD	potential difference
PKC	protein kinase C
rENaC	rat epithelial sodium channel
RVD	regulatory volume decrease
RVI	regulatory volume increase
R-PIA	(-)-N^6-(R-phenylisopropyl)adenosine
TEA	tetraethylammonium
TG	thapsigargin
UV	ultra violet light
UTP	uridine triphosphate

Defined symbols and units

T	absolute temperature; K
pH	acidity
K	apparent equilibrium constant for the reaction between Ins(3,4,5,6)P_4 and its intracellular target
σ_0^2	background variance; pA^2; picoamperes squared
c	concentration of Ins(3,4,5,6)P_4; μM; micromolars
a	cooperativity factor; dimensionless
θ_1, θ_2	corner frequencies; Hz; Hertz
I_{base}	current unaffected by Ins(3,4,5,6)P_4; pA; picoamperes
$[x]_o$, $[x]_i$	extra- and intracellular concentrations for ions; mM; millimolars
F	Faraday's constant (9.648 x 10^4 C mol^{-1})
f	frequency; Hz; Hertz
S_1, S_2	frequency plateaux; pA^2/Hz
R	gas constant (8.315 J K^{-1} mol^{-1})
InsP_3R	inositol 1,4,5-trisphosphate receptor
x	ions
I_{max}	maximum current blocked by Ins(3,4,5,6)P_4; pA; picoamperes
V_m	membrane potential; mV; millivolts
N	number of channels
P_o	open probability of channels
E	reversal potential; mV; millivolts
γ	single-channel conductance; pS; picosiemens
I	total membrane current; pA; picoamperes
σ^2	total variance; pA^2; picoamperes squared
Z	valency

cell/ml	concentration; number of cells per millilitre
nM	concentration; nanomolars
U/ml	concentration; units of protein per volume in millilitres
W/V	concentration; weight in grams per volume in litre
mg/ml	concentration; weight in milligrams per volume in millilitre
KHz	frequency; kilohertz
g	gravitational acceleration; 9.81 ms^{2}
mm	length; millimetres
nm	length; nanometres
MΩ	resistance; megaohms
°C	temperature; degrees Celsius
ms	time; milliseconds
s	time; seccond

1. GENERAL INTRODUCTION

1.1 Ion transport in secretory epithelia

Epithelial tissues consist of closely packed cells forming flat sheets with single layer or multilayer thickness. They are usually found at the interface between the organism and the external environment, as in the airway epithelium, although the external environment may be extensively internalized and fluid-filled, as in the gut or bladder epithelia. The location of epithelial cells implies their importance in protecting the relevant body parts, and in providing transport of material to and from the external environment by absorption, filtration and secretion.

The normal physiological functions of epithelial cells are highly dependent on their abilities to perform polarized transport of substances such as ions, water, nutrients and substances for excretion. This polarized transport is achieved by having different membrane components on the apical (mucosal) and the basolateral (serosal) membranes. The cellular mechanism for Cl^- secretion is shown in Fig. 1.1 (Frizzell & Halm 1990). Chloride enters the basolateral membrane of the secretory cell via an electrically neutral Na-K-2Cl cotransport system (Frizzell & Halm 1990; Welsh 1987). Sodium ions enter the cell down a favourable electrochemical gradient, providing energy to drive Cl^- against its electrochemical gradient. Chloride ions accumulate within the secretory cell above their electrochemical equilibrium and exit passively

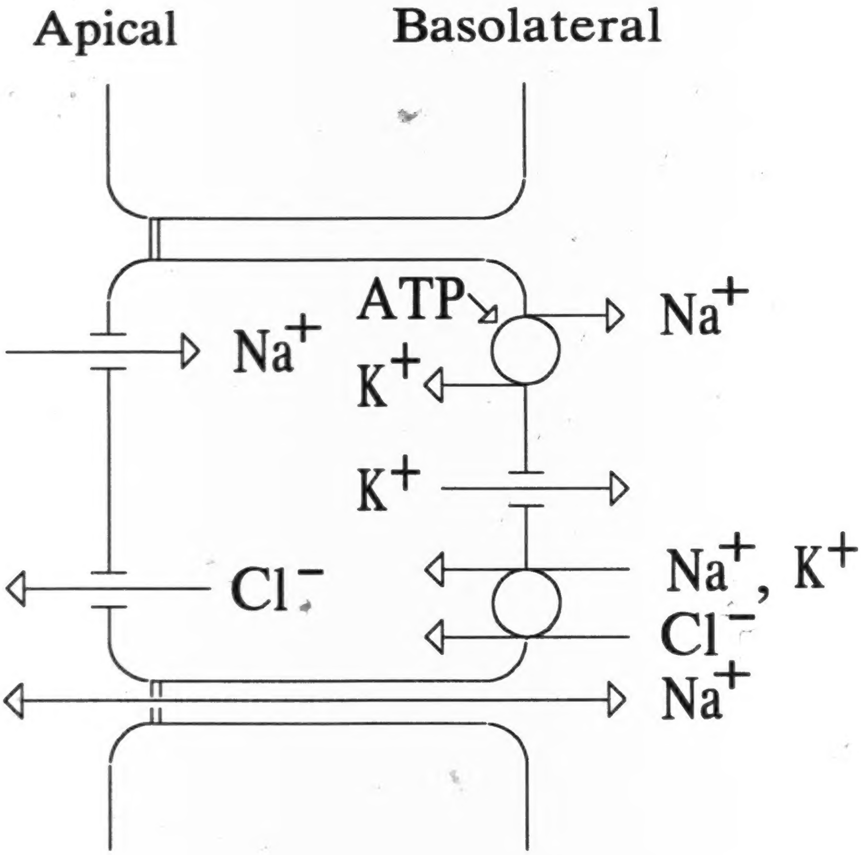
Apical
Basolateral
ATP
Na+
Na+
K+
K+
Cl−
Na+, K+
Cl−
Na+

Figure 1.1. A model of ion transport in a secretory epithelial cell.

down this gradient through an apical membrane Cl^- channel. This Cl^- secretion creates a transepithelial potential difference (PD), with the mucosal side being more negative than the serosal side. Hence, the transport is electrogenic in nature. Intracellular cAMP & Ca^{2+} and volume changes regulate this apical conductance and represent the principle control mechanisms for Cl^- secretion (Anderson *et al.*, 1992).

Sodium ions enter the cell via the Na-K-2Cl cotransport and exit across the basolateral membrane by means of the Na^+-K^+-adenosinetriphosphatase (ATPase) pump (Frizzell & Halm 1990; Welsh 1987). This pump maintains the Na^+ gradient that is necessary for Cl^- entry and hence Cl^- secretion. However, Na^+ ions can also cross the apical membrane through Na^+ channels. As well as this apical Na^+ current, a paracellular Na^+ flow can be driven by the epithelial PD if there is Na^+ permeability between the cells.

The potassium ions that are brought into the secretory cell by the Na-K-2Cl cotransport and Na^+-K^+-ATPase pump exit the cell passively through basolateral membrane K^+ channels (Frizzell & Halm 1990; Welsh 1987). Increasing this basolateral K^+ current would hyperpolarize the secretory cell, providing a greater electrical gradient for Cl^- secretion.

Any defects in these membrane components can cause malfunctioning of the epithelium. Changes in apical membrane Cl^- channel function can cause abnormal chloride secretion in several epithelia. Excess Cl^- secretion in the intestinal epithelium promotes diarrhoea (Kanwar *et al.*, 1995) while reduced Cl^- secretion in the airway epithelium induces thicker mucus, as occurs in cystic fibrosis (CF) patients (Quinton 1990). A better understanding of the control mechanisms of Cl- secretion is needed to gain insight into these pathological conditions.

1.2 Cystic fibrosis

Electrolyte transport across epithelial cells requires a set of components in both the apical and basolateral membrane. Defects in any of these transport components can cause electrolyte imbalance and therefore abnormal epithelial function. Cystic fibrosis (CF) is a conspicuous example of such abnormal electrolyte transport, which results in severe pathological conditions. CF is recognized as the most common fetal autosomal recessive genetic disease affecting the Caucasian population (Quinton 1990). In the United States alone, CF affects about 1 in 2000 Caucasians. CF affects epithelia in many different systems including the salivary glands, reproductive organs, intestine, sweat glands, pancreas, and airway (Quinton 1990). Despite the diversity of the affected organs, the overall symptoms in these systems are primarily due to an impaired apical cAMP-regulated Cl^- permeability (Boucher *et al.*, 1989).

With modern therapies, the major problem for CF patients is an impaired airway function, which contributes to the major causes of morbidity and mortality in CF. In the CF airway there is an increase in Na^+ reabsorption, as well as a reduction in Cl^- secretion (Boucher *et al.*, 1986; Quinton 1983). Consequently, airway mucus becomes thick and tenacious, causing a major problem in ciliary clearance (Quinton 1990). The viscous mucus then provides a substrate for chronic infection with bacteria like *Pseudomonas aeruginosa* and *Staphylococcus aureus*, which are often seen in the CF airway (Quinton 1990). However, it is likely that bacterial infections can also contribute to the formation of viscous mucus in CF. Chronic infections progressively destroy the parenchyma of the lung and lead to complications like pulmonary hypertension and ventricular hypertrophy (Quinton 1990). These complications ultimately lead to cardiorespiratory failure and death.

The gene responsible for CF has been identified on human chromosome 7 (Tsui *et al.*, 1985; Wainwright *et al.*, 1985; White *et al.*, 1985), and subsequently cloned (Riordan *et al.*, 1989; Rommens *et al.*, 1989). The CF gene product has been named the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is predicted to have 1480 amino acids and a calculated molecular weight of about 170 kDa (Riordan *et al.*, 1989). The predicted structure for CFTR consists of two repeated motifs, each with a hydrophobic domain (HD) and a nucleotide-binding domain (NBD) (Riordan *et al.*, 1989). Each of these hydrophobic domains is comprised of six α-helical transmembrane spanning segments. Downstream of each HD is the NBD, which consists of 150 amino acids, and shows consensus sequences for ATP binding and/or hydrolysis with a ß-sheet structure.

The overall structural organization and the NBD sequence for CFTR strongly resemble proteins from a family of transport proteins called the ATP-binding cassette (ABC) transporters (Riordan *et al.*, 1989). This ABC superfamily contains a large array of transporters with different functions. However, the predicted overall structure for CFTR also suggests a regulatory domain (RD) between the two repeated motifs that is unique to CFTR and not found in the other members of the ABC superfamily (Riordan *et al.*, 1989). This RD contains 241 amino acids, 69 of which are polar residues arranged in alternating clusters of positive and negative charges. Moreover, this RD has several consensus phosphorylation sequences for PKA and PKC (Riordan *et al.*, 1989). Mounting evidence has supported the idea that CFTR functions as a cAMP-regulated Cl^- channel (Anderson *et al.*, 1991b; Bear *et al.*, 1992; Dalemans *et al.*, 1991; Haws *et al.*, 1992; Tabcharani *et al.*, 1991).

More than 400 mutations have been localized on the CF gene since its discovery. Most of these mutations occur within the two NBDs and contribute to varying degrees to the clinical manifestations of CF. Among these mutations, a single deletion of a phenylalanine residue at position 508 (ΔF508) in NBD1 is the most common mutation, representing about 70% of the total mutations in CF (Kerem *et al.*, 1989). Patients with homozygous ΔF508 exhibit the most severe form of CF, with extreme pulmonary distress and pancreatic insufficiency, whereas heterozygous ΔF508 carriers are much less affected. However, there is no clear link between the CF genotypes and the clinical phenotypes since many CF patients carry more than one mutation.

Treatments for CF are generally divided into two categories: long term treatments which tackle the expression or trafficking defects of CFTR, and more symptom-relief oriented treatments that involve pharmacological modulation of the overall electrolyte transport in the affected organs. However, these treatments are intended for the more severe clinical manifestation in the airway, since enzyme supplements can correct much of the pancreatic insufficiency.

Long term cures for CF have recently been focused on gene therapy of the airway epithelium. Adenovirus has been tested extensively for its potential role as a viral vector for introducing CFTR into CF hosts (Smith 1995). This virus contains a small, double stranded DNA. It is usually associated with mild disease, and its molecular biology is well understood (Berkner 1988). The transfection rate of adenovirus with a normal CFTR gene has been shown to be efficient (Zabner *et al.*, 1994). However, gene transfer with adenovirus induces inflammatory responses in the hosts and this could contribute to its transient transfer properties (Yang *et al.*, 1994). Trials are currently underway to test the safety of repeated administrations of adenovirus encoded with CFTR (Zabner *et al.*, 1996). Besides viral vector systems, non-viral systems like cationic lipids and receptor-mediated gene transfer have also been explored (Smith 1995). Receptor-mediated gene transfer systems allow specific cells to be targeted. These non-viral systems are promising because they have minimal immunological responses, and are relatively nontoxic.

Much treatment for CF has been concentrated on symptomatic relief, especially for easing the accumulation of viscous mucus in the airway. Increased mucus viscosity in the airway is the direct result of increased Na^+ reabsorption and reduced Cl^- secretion, reducing the water content of airway mucus (Boucher *et al.*, 1986; Quinton 1983). Furthermore, macromolecules like DNA are released from the damaged cells and further increase mucus viscosity (Picot *et al.*, 1978). For patients with severe CF symptoms, mechanical assistance for mucous clearance and repeated antibiotic treatment are common. Pharmacological modulation of electrolyte transport and therefore mucus viscosity has also been suggested as part of the CF management strategy. Amiloride is a potent blocker of Na^+ channels in both normal and CF epithelia (Boucher *et al.*, 1986). The use of aerosol amiloride in CF patients has been promising, with the rheology of airway secretions becoming normalized (Knowles *et al.*, 1990). However, more studies on amiloride metabolism and safety are needed. Similarly to amiloride, the use of DNase has shown to reduce viscoelasticity of CF sputum, offering another means to improve overall mucociliary clearance (Hodson & Shah 1995). Long term safety of DNase treatment has yet to be determined.

Another possible pharmacological intervention is to stimulate alternative Cl^-

conductances to the cAMP-regulated CFTR. A Ca^{2+}-dependent Cl^- conductance was observed in both normal and CF airway and pancreatic cells (Anderson & Welsh 1991; Boucher *et al.*, 1989; Clarke *et al.*, 1994), but not in intestinal epithelia of CF origin (Anderson & Welsh 1991; Berschneider *et al.*, 1988; Clarke *et al.*, 1994). Expression of this alternative Cl^- conductance might therefore compensate for the loss of CFTR and has been suggested to attribute to the varying degrees of CF manifestation (Clarke *et al.*, 1994). Agonists like ATP and UTP which increase cellular Ca^{2+} via a PLC-dependent pathway upon receptor activation (see Section 1.5, Purinergic Receptors, for details) are being considered for their potential therapeutical use (Knowles *et al.*, 1995). These nucleotides have been shown to increase Cl^- conductance in CF epithelia (Clarke & Boucher 1992; Mason *et al.*, 1991; Stutts *et al.*, 1994). However, prolonged use of these PLC-dependent agonists is limited by feedback control (see Section 1.6, PLC-dependent inositol phosphate cycle), and a better understanding of this pathway is needed to improve its efficacy in CF treatment (Jiang *et al.*, 1993; Kachintorn *et al.*, 1993; Traynor-Kaplan *et al.*, 1994; Vajanaphanich *et al.*, 1994).

1.3 Ion channels in epithelia

Transepithelial ion transport is a well coordinated process that requires functional transport elements in both the apical and basolateral membranes, including ion channels. There are several types of ion channels in epithelial cells, including

Na^+, K^+, Cl^- and the less well known Ca^{2+} channels. These ion channels have a range of biophysical properties and are regulated by a variety of cellular mechanisms. Each type of channel can be further classified, based on its single-channel properties, blocker sensitivity, and regulatory properties. In this section I will focus on the Na^+, K^+, and Cl^- channels, because these are the three crucial components in most transport schemes.

1.3.1 Sodium channels

Unlike the Na^+ channels of excitable tissues, the Na^+ channels in epithelial cells are not voltage-sensitive, and not sensitive to toxins such as tetrodotoxin. Instead they have high affinity to the diuretic blocker 3,5-diamino-N-(aminoiminomethyl)-6-chloropyrazinecarboxamide, which is more commonly known as amiloride. The single-channel conductance of the amiloride-sensitive Na^+ channel has been reported to range from ~5-25 pS, with variable Na^+ selectivity (Palmer 1992).

Amiloride-sensitive Na^+ channels are regulated by a variety of hormones, in particular those that play important roles in electrolyte balance, such as vasopressin and aldosterone (Benos *et al.*, 1995). The overall effects of these hormones on Na^+ channel are via regulation of either channel kinetics, channels number or both. Activation of the amiloride-sensitive Na^+ channel was shown to involve direct PKA phosphorylation of the channel protein (Oh *et al.*, 1993) or phosphorylation of actin filaments (Prat *et al.*, 1993). However, a rise in cellular Ca^{2+} level was demonstrated to have an inhibitory effect on the amiloride-sensitive Na^+ channel (Silver *et al.*, 1993).

Our understanding of the structure and function of amiloride-sensitive Na^+ channels has now been facilitated by the purification and molecular cloning of channel proteins. The most extensively studied and characterized Na^+ channel protein was purified from A6 cells and bovine renal papilla (Benos *et al.*, 1987). This protein has a molecular mass of about 700 KDa and is comprised of six associated proteins. These polypeptides have molecular masses of about 300, 150, 95, 70, 55 and 40 KDa, and each of them shows different binding affinity to various cellular proteins. The 300 KDa polypeptide is the only substrate for PKA, whereas PKC has affinity for both the 150 and 55 KDa subunits (Oh *et al.*, 1993). Besides being a PKC substrate, the 150 KDa peptide is the only subunit that shows affinity to amiloride (Benos *et al.*, 1987). The 95 KDa subunit can be methylated by carboxymethyltranferase and has been suggested to play a role in channel regulation by aldosterone (Sariban-Sohraby *et al.*, 1984). Similarly to the 95 KDa subunit, the 70 KDa has been suggested to be involved in aldosterone-dependent channel activation (Szerlip *et al.*, 1991). The 40 KDa subunit, has been identified as the α subunit of a G-protein and shown to be involved in the cyclic nucleotide regulation of Na^+ channels (Ausiello *et al.*, 1992).

When incorporated into lipid bilayers, the 700 KDa protein exhibited a single-channel conductance of about 9-60 pS (Oh & Benos 1993). This isolated channel protein showed a high selectivity for Na^+ and affinity to amiloride. This channel was regulated by phosphorylation, either being stimulated or inhibited by PKA or PKC, respectively (Oh *et al.*, 1993). However, when only the 150 KDa was incorporated into liposomes, a channel of about 5 pS was observed (Sariban-Sohraby *et al.*, 1992). It was therefore suggested that the 150 KDa peptide represents the conductive pore of the amiloride-sensitive channel.

An amiloride-sensitive Na^+ channel, comprising α, ß, and γ subunits has been recently cloned from rat colon (rENaC) (Canessa *et al.*, 1993 & 1994; Lingueglia *et al.*, 1993). The α, ß, and γ subunits have calculated molecular masses of 78, 72, and 75 KDa, respectively and are widely distributed in Na^+ absorptive epithelia like the kidney, colon and lung. These subunits were shown to have a high degree of homology to each other. rENaC was predicted to consist of two transmembrane spanning regions and a long extracellular loop (Canessa *et al.*, 1993), but the stoichiometry for functional rENaC is not known.

When all three subunits were expressed in oocytes, rENaC showed high sensitivity to amiloride and high selectivity to Na^+ (Canessa *et al.*, 1994; Lingueglia *et al.*, 1993). The single-channel conductance of rENaC was ~5 pS (Canessa *et al.*, 1993; Lingueglia *et al.*, 1993). Similarly to the 150 KDa subunit from the 700 KDa protein, only αrENaC was reported to show affinity to amiloride and channel activity when expressed in oocytes (Canessa *et al.*, 1993 & 1994). However, the current induced by αrENaC alone was significantly smaller in the absence of the ß and γ subunits (Canessa *et al.*, 1994). It was proposed that both ß and γ subunits had a regulatory role and were required for full activation of rENaC. The exact relationship between rENaC and the 700 KDa protein is not known. However, antibodies against αrENaC were reported to recognize both the 95 and 70 KDa peptides from the 700 KDa protein (Benos *et al.*, 1995).

1.3.2 Potassium channels

Potassium channels represent a diverse group of ion channels in epithelial cells. They are crucial for maintaining the membrane potential and driving force for Cl^- secretion. The most studied K^+ channels in epithelia are the voltage- and Ca^{2+}-dependent K^+ channels.

1.3.2.1 Voltage-dependent K^+ channels

The voltage-dependent inwardly rectifying (VDIR) K^+ channel has been widely observed in renal epithelial cells (Ho *et al.*, 1993; Kubo *et al.*, 1993; Parent *et al.*, 1988; Zhou *et al.*, 1994). Membrane hyperpolarization activates the VDIR K^+ channels and causes a large inward K^+ current. Single-channel conductance for the VDIR K^+ channels was reported to range from ~20-55 pS. This channel showed voltage-dependent blockade by extracellular Ba^{2+}, Cs^+, Na^+, and K^+ (Kubo *et al.*, 1993; Zhou *et al.*, 1994), but not to external tetraethylammonium (TEA) (Ho *et al.*, 1993; Zhou *et al.*, 1994). However, intracellular Ba^{2+} blockade was also reported (Ho *et al.*, 1993).

The epithelial VDIR K^+ channel has a distinct putative structure that is different from the inward rectifier of the superfamily of voltage-gated channels. The VDIR K^+ channel protein has recently been cloned, with predicted molecular mass of 45 KDa (Ho *et al.*, 1993). Unlike the inward rectifier in neuronal systems, the epithelial VDIR K^+ channel was predicted to have only two putative membrane spanning segments (Ho *et al.*, 1993; Kubo *et al.*, 1993; Zhou *et al.*, 1994). However, the predicted ion conducting region (S5-S6 linker or H5 region) of the classic inward rectifier was conserved in the epithelial VDIR K^+ channel (Ho *et al.*, 1993; Kubo *et al.*, 1993; Zhou *et al.*, 1994). It is likely that the epithelial VDIR K^+ channel represents a new class of voltage-dependent inwardly rectifying K^+ channels.

Another voltage-dependent K^+ channel with distinct properties has been identified in renal and airway epithelia (DeCoursey *et al.*, 1988; Volk *et al.*, 1994). Unlike the VDIR K^+ channel, this K^+ channel was shown to activate by membrane depolarizations greater than -30 mV (Volk *et al.*, 1994). A time- and voltage-dependent slow inactivation was also reported in this K^+ channel. The depolarization-activated K^+ current was blocked by external TEA, 4-aminopyridine

(4-AP) and charybdotoxin (ChTX) (DeCoursey *et al.*, 1988; Volk *et al.*, 1994). Such biophysical and pharmacological properties suggest similarities between the depolarization-activated epithelial K^+ channel and the delayed rectifier reported in neuronal systems. Molecular identification of the depolarization-activated epithelial K^+ channel have supported its close linkage to the delayed rectifier (Desir *et al.*, 1992; Volk *et al.*, 1994).

1.3.2.2 Ca^{2+}-dependent K^+ channels

Ca^{2+}-dependent K^+ channels are also well studied in epithelia and show diverse biophysical properties. A Ca^{2+}-dependent inwardly rectifying (CaIR) K^+ channel was reported in various epithelia (Devor & Frizzell 1993; Roch *et al.*, 1995; Wang 1994; Welsh & McCann 1985). Unlike the VDIR K^+ channel, the CaIR K^+ channel was activated only by cytosolic Ca^{2+} (Devor & Frizzell 1993; Roch *et al.*, 1995; Welsh & McCann 1985) and was insensitive to membrane potential (Wang 1994). The single-channel conductance for the CaIR K^+ channel was reported to range from ~30-40 pS (Devor & Frizzell 1993; Roch *et al.*, 1995; Wang 1994; Welsh & McCann 1985). The active CaIR K^+ channel was shown to be sensitive to extracellular TEA and ChTX (Devor & Frizzell 1993; Roch *et al.*, 1995) and intracellular 4-AP, quinine, ATP and Ba^{2+} blockade (Devor & Frizzell 1993; Roch *et al.*, 1995; Wang 1994). The physiological function of the CaIR K^+ channel might involve volume regulation. Swelling-activated, Ca^{2+}-dependent K^+ conductances has been reported in several epithelial cells (Adorante & Cala 1995; Diener *et al.*, 1992; Nilius *et al.*, 1995a; Weiss & Lang 1992), with single-channel properties similar to the CaIR K^+ channel (Nilius *et al.*, 1995a; Weiss & Lang 1992).

Another Ca^{2+}-dependent epithelial K^+ channel had a single-channel conductance of 150-300 pS, and was termed the maxi K^+ channel (Kunzelmann *et al.*, 1989a; Ridge *et al.*, 1996; Turnheim *et al.*, 1989). In contrast to the CaIR K^+ channel, activation of the maxi K^+ channel was demonstrated to be both voltage-dependent and Ca^{2+}-dependent. Intracellular Ca^{2+} further increased channel open probability at constant holding voltage without rectification. Blockade by Ba^{2+}, quinine and TEA were reported from both sides, whereas ATP and ChTX inhibited the maxi K^+ channel from only the cytoplasmic and extracellular sides, respectively (Kunzelmann *et al.*, 1989b; Ridge *et al.*, 1996; Turnheim *et al.*, 1989). A swelling-activated K^+ channel with similar properties was reported in some cell types (Edelman et al. 1995; Park *et al.*, 1994).

Molecular characterization of the epithelial maxi K^+ channel is not available. However, a similar maxi K^+ channel has recently been purified and cloned from smooth muscle (Kaczorowski *et al.*, 1996). The maxi K^+ channel from smooth muscle consisted of two subunits, α and ß, with a molecular weight of 60-70 and 31 KDa, respectively. The α subunit had ten predicted transmembrane segments and was a member of the *slo* Ca^{2+}-activated K^+ channel gene family. Functional K^+ channels could be formed by the α subunit alone, but inclusion of the ß subunit increased voltage-sensitivity (Kaczorowski *et al.*, 1996).

1.3.3 Chloride channels

Chloride channels in epithelia have received a great deal of attention because of their probable roles in cystic fibrosis. Apical Cl^- channels play an important role in Cl^- secretion and therefore fluid balance. Understanding these apical Cl^- channels might provide a better insight into CF management.

1.3.3.1 cAMP-activated Cl^- channels

The most studied ion channel in epithelial cells are the cAMP-regulated Cl^- channels because of the clinical manifestation of cystic fibrosis (CF) which are typified by an abnormal apical Cl^- permeability (Quinton 1983). The CF gene product, the cystic fibrosis transmembrane conductance regulator (CFTR) is a 3',5'-cyclic adenosine monophosphate (cAMP)-regulated Cl^- channel (see Section 1.2 Cystic Fibrosis). When CFTR cDNA was expressed in cells with little or no endogenous CFTR, cAMP induced a linear whole-cell current, with no obvious voltage-dependent or time-dependent activation and inactivation (Dalemans *et al.*, 1991; Haws *et al.*, 1992; Tabcharani *et al.*, 1991). The single-channel conductance of CFTR is about 5-10 pS, with a halide permeability sequence $Br^- > Cl^- > I^-$ and insensitivity to DIDS blockade (Dalemans *et al.*, 1991; Haws *et al.*, 1992; Tabcharani *et al.*, 1991). These properties of recombinant CFTR are similar to those recorded from cells expressing endogenous CFTR (Bear & Reyes 1992; Tabcharani *et al.*, 1990).

Activation of CFTR requires PKA phosphorylation on its regulatory domain (RD) (Dalemans *et al.*, 1991; Haws *et al.*, 1992; Tabcharani *et al.*, 1991). There are ten high affinity consensus sequences for PKA on the RD (Riordan *et al.*, 1989) but none of them were shown to be essential, and phosphorylation at any single site was enough for CFTR activation (Chang *et al.*, 1993). Besides PKA, consensus sequences for PKC were also identified on RD (Riordan *et al.*, 1989) and PKC phosphorylation was demonstrated to potentiate the subsequent action of PKA on CFTR activation (Tabcharani *et al.*, 1991). In addition to PKA phosphorylation, ATP binding to the two nucleotide binding domains is also required to maintain CFTR activity (Anderson *et al.*, 1991a). However, the exact nature of such ATP interaction and channel gating is controversial (Anderson *et al.*, 1991a; Quinton & Reddy 1992).

Similarly to CFTR, another cAMP-regulated Cl^- channel has been reported in many different epithelia (Anderson *et al.*, 1992). This Cl^- channel shows outward rectification under symmetrical Cl^- solutions and is therefore called the outwardly rectifying Cl^- channel (ORCC). Single-channel recording of ORCC shows a nonlinear current-voltage relationship, with a chord conductance of ~40-50 pS for outward current. Unlike CFTR, ORCC is more permeable to I^-, with an anion permeability sequence of $I^- > Cl^- > Br^-$.

A wide range of physiological compounds and physical manoeuvres can activate ORCC. ORCC was demonstrated to be activated by PKA- and PKC-dependent phosphorylation (Welsh *et al.*, 1989). However, the effect of PKC on ORCC was shown to be Ca^{2+}-dependent, with reduced PKC activation at intracellular Ca^{2+} >150 nM (Li *et al.*, 1989). ORCC was reported to be voltage-dependent, with channel activation occurring at strong depolarizing voltages (> 50 mV) for seconds to minutes (Welsh *et al.*, 1989). Other manipulations to activate ORCC included exposing the channel to high ion content solutions, trypsin and bath solution at 37°C (Kunzelmann *et al.*, 1989b; Welsh *et al.*, 1989). Unlike CFTR, ORCC shows sensitivity to DIDS (Anderson *et al.*, 1992) and other compounds such as arachidonic acid and leukotrienes (Hwang *et al.*, 1990). The differences in their biophysical properties and blocker sensitivities allows a clear separation of CFTR and ORCC.

The cAMP regulation, but not the voltage-dependent activation of ORCC is also defective in CF epithelia (Schoumacher *et al.*, 1987). The exact relationship between defective CFTR and ORCC in CF epithelia is not clear. However, the defective cAMP regulation of ORCC in CF epithelia was shown to be normal in the presence of functional CFTR (Egan *et al.*, 1992; Gabriel *et al.*, 1993), suggesting a regulatory role, as well as a channel function for CFTR. Extracellular ATP was shown to increase ORCC activity in CF epithelia via a surface purinergic receptor-mediated, Ca^{2+}-independent pathway (Stutts *et al.*, 1992). Recent studies demonstrated that CFTR functions as both Cl^- and ATP channels (Reisin *et al.*, 1994; Schwiebert *et al.*, 1995). This has led to the proposal that CFTR regulates ORCC via an autocrine mechanism in which CFTR conducts ATP out of the cell and ATP activates ORCC through surface purinergic receptors (Schwiebert *et al.*, 1995). However, such CFTR-mediated ATP conductance was not observed in other studies (Grygorczyk *et al.*, 1996; Li *et al.*, 1996; Reddy *et al.*, 1996).

1.3.3.2 Ca^{2+}-dependent Cl^- channels

Intracellular Ca^{2+} is another important signalling molecule for the regulation of epithelial Cl^- channels. However, the apical Ca^{2+}-dependent Cl^- channel is not as well known as CFTR or ORCC. This Ca^{2+}-dependent Cl^- channel remains intact in CF airway and pancreatic tissues (Anderson & Welsh 1991; Boucher *et al.*, 1989; Chan *et al.*, 1992; Clarke *et al.*, 1994) but not in CF intestinal epithelia (Anderson & Welsh 1991; Berschneider *et al.*, 1988; Clarke *et al.*, 1994), questioning the existence of endogenous Ca^{2+}-dependent Cl^- channels. However, a Ca^{2+}-dependent Cl^- conductance was demonstrated in non-polarized intestinal epithelial cells grown on impermeable supports (Anderson & Welsh 1991). An observed transepithelial Cl^- secretion in polarized intestinal cells induced by Ca^{2+} agonists was suggested to be the result of increased Ca^{2+}-activated K^+ channel activity in the basolateral membrane. Enhanced K^+ conductance would then hyperpolarize the cells and increase the driving force for Cl^- current via some pre-opened apical Cl^- channels. The exact nature of such differences in the expression of the Ca^{2+}-dependent Cl^- channels in differentiated and non-polarized cells are not clear. The lack of Ca^{2+}-dependent Cl^- secretion in CF intestinal cells might be due to the absence of functional Cl^- channels in those cells. Despite its ambiguous role in intestinal epithelia, the Ca^{2+}-dependent Cl^- conductance remains intact in CF airway epithelia, and has been suggested as an alternative current to compensate for the loss of cAMP-activated Cl^- current.

Typical whole-cell Ca^{2+}-dependent Cl^- current is quite different from the cAMP-activated Cl^- current. The Ca^{2+}-dependent Cl^- current shows strong outward rectification, with time-dependent activation at depolarizing potentials (Anderson & Welsh 1991; Cliff & Frizzell 1990; Wagner *et al.*, 1991; Worrell & Frizzell 1991). Time-dependent inactivation at hyperpolarizing potentials was demonstrated in T84 cells (Cliff & Frizzell 1990) but not in airway epithelia (Anderson & Welsh 1991; Wagner *et al.*, 1991). Similarly to ORCC, the Ca^{2+}-dependent Cl^- current was shown to be sensitive to DIDS blockade, and higher permeability to I^- than to Cl^- (Anderson & Welsh 1991). Activation of the Ca^{2+}-dependent Cl^- current was demonstrated by mutltifunctional Ca^{2+}/calmodulin-dependent protein kinase II (CaCMKII) phosphorylation, but not by PKC (Wagner *et al.*, 1991; Worrell & Frizzell 1991; Xie *et al.*, 1996).

In contrast to CFTR, little is known about the biophysical and molecular properties of the Ca^{2+}-dependent Cl^- channel underlying the observed whole-cell current. Cell-attached or excised-patch recordings from various epithelial cells have reported a single-channel conductance of the Ca^{2+}-dependent Cl^- channel ranging from 3-50 pS (Frizzell *et al.*, 1986; Marunaka & Eaton 1990; Morris & Frizzell 1993; Nilius *et al.*, 1995b). However, no intrinsic outward rectification was observed in some of these Ca^{2+}-dependent Cl^- channels (Frizzell *et al.*, 1986; Nilius *et al.*, 1995b).

The molecular structure of the Ca^{2+}-dependent Cl^- channel remains elusive. However, a Cl^- channel with a molecular weight of 140 KDa has recently been purified from bovine trachea (Fuller *et al.*, 1994). When incorporated into bilayers, this Cl^- channel was insensitive to PKA activation, but was activated by raising Ca^{2+} on the intracellular side to 3 μM or above (Fuller *et al.*, 1994). The single-channel conductance of the 140 KDa Cl^- channel was about 25-30 pS, with a linear current-voltage relationship under symmetrical Cl^- conditions. Similarly to the other Ca^{2+}-dependent Cl^- channels, the 140 KDa Cl^- channel was also sensitive to DIDS blockade. The Ca^{2+} dependence of channel activation for the 140 KDa Cl^- channel was drastically reduced in the presence of CaMKII (~ 600 nM), suggesting a facilitatory role for CaMKII phosphorylation in channel activation. However, further increase in Ca^{2+} (> 1 μM) in the presence of CaMKII caused a Ca^{2+}-dependent block of channel activities.

1.3.3.3 Swelling-activated Cl^- channels

Volume change is also an important physiological factor for regulating transepithelial Cl^- secretion. At least three proteins have been associated with swelling-activated Cl^- conductances, the pI_{Cln}, ClC-2, and P-glycoproteins (Gill *et al.*, 1992; Paulmichl *et al.*, 1992; Thiemann *et al.*, 1992; Valverde *et al.*, 1992). Each of these protein molecules is associated with a distinct swelling-activated Cl^- conductance and a detailed discussion is presented in Section 1.4, Volume Regulation.

1.3.3.4 Other epithelial chloride channels

The Cl^- channels discussed above do not represent the total Cl^- channels in epithelia. In fact, a variety of other Cl^- channels have been identified in different epithelial cells. In the airway epithelium, at least three other types of Cl^- channels, with single-channel conductances of 4 pS, 10 pS and 20 pS have been shown to have completely different biophysical properties to the Cl^- channels described here (Duszyk et al., 1989 & 1992; Wilk-Blaszczak et al., 1992). These three types of Cl^- channels all had linear current-voltage relationships and were insensitive to membrane potential. Similarly to CFTR and ORCC, the 20 pS channel was also activated by PKA phosphorylation and shown to be defective in CF airway epithelia (Duszyk et al., 1989). A similar 20 pS Cl^- channel with an ohmic current-voltage relationship has also been identified in CFPAC-1 cells (Duszyk et al., 1993). In addition to the 20 pS

Cl$^-$ channel, a voltage-dependent Cl$^-$ channel with large conductance (~390 pS) and complex kinetic behavior has also been reported in CFPAC-1 cells (Duszyk et al., 1993). Chloride channels with similar conductance have been demonstrated in other epithelia (Hanrahan *et al.*, 1985; Schwiebert *et al.*, 1994). Besides their diverse biophysical properties, epithelial Cl$^-$ channels can be regulated in several ways, including G-proteins (Duszyk et al., 1995; Schwiebert et al., 1990), pH (Cuppoletti *et al.*, 1993), phosphorylation and Ca^{2+}-dependent mechanisms. This diversity of epithelial Cl$^-$ channel types emphasizes the important physiological roles that chloride transport must play in epithelial tissues.

Among the known Cl$^-$ channels, the voltage-gated Cl$^-$ channels of the ClC gene family represent a diverse and important group. Similarly to CFTR, defective ClC Cl$^-$ channels are also linked to various diseases such as myotonia and Dent's disease (Jentsch 1996). Eleven ClC Cl$^-$ channels have been cloned from various tissues (Table 1.3.1). All members of the ClC family share a common topology, consisting of twelve transmembrane domains (TM1-TM12), a conserved cytosolic TM13 domain, and cytosolic N- and C-terminals (Jentsch 1996). However, a recently revised model has suggested that the TM4 domain does not transverse the plasma membrane and most likely represents an extracellular loop between TM3 and TM5. In general, the ClC Cl$^-$ channels are divided into three subfamilies, based on sequence similarities with other members of the ClC family (Jentsch 1996). Members of the first subfamily, ClC-0, ClC-1, ClC-2, ClC-K1, and ClC-K2, show 40% to 80% amino acid identity to each other. The second subfamily includes ClC-3, ClC-4 and ClC-5, which share ~80% amino acid identity among its members. The last branch of the ClC Cl^- channels consists of ClC-6 and ClC-7, which have ~45% amino acid agreement and even less (~20-30%) with the other two subfamilies.

Despite the similarities in their overall topology and amino acid sequences, each member of the ClC family exhibits different biophysical properties, blocker sensitivities and regulatory mechanisms. The characteristics of some of the ClC members are summarized in Table 1.3.2. Unlike the ClC Cl^- channels listed in Table 1.3.2, there are no successful reports of functional expression studies on ClC-4, ClC-6 or ClC-7 (Jentsch 1996). The diversity of ClC Cl^- channel types further emphasizes the important physiological roles of Cl^- channels in cellular functions.

Channel	Number of Amino acids	Calculated size (KDa)	Expression	References
ClC-0	805	89	Electric organ	Jentsch *et al.*, 1990
ClC-1	994	110	Skeletal muscle	Steinmeyer *et al.*, 1991
ClC-2	907	99	Ubiquitous	Thiemann *et al.*, 1992
ClC-2G	898	98	Gastric mucosa	Malinowska *et al.*, 1995
ClC-3	760	84	Brain, kidney	Kawasaki *et al.*, 1994
ClC-4	760	84	Skeletal muscle, Kidney, Brain	van Slegtenhorst *et al.*, 1994
ClC-5	746	83	Kidney, Brain, Lung	Steinmeyer *et al.*, 1995 Sakamoto *et al.*, 1996
ClC-6	869	97	Ubiquitous	Brandt & Jentsch 1995
ClC-7	803	89	Ubiquitous	Brandt & Jentsch 1995
ClC-K1	686	76	Kidney	Uchida *et al.*, 1993
ClC-K2	687	76	Kidney	Adachi *et al.*, 1994

Table 1.3.1. Molecular properties of the cloned ClC Cl^- channels

Channel	Rectification & Anion Selectivity	Voltage Dependence	Channel Conductance (pS)	Blocker Sensitivity	Regulation	Functions
ClC-0	Linear $Cl^->Br^->I^-$	Fast gating at + V_m Slow gating at - V_m	~10	DPC, 9-AC, DIDS	Voltage	Stablization of Vm
ClC-1	Inward $Cl^->Br^->I^-$	Opens at + V_m	~1	9-AC, DPC	Voltage	Stablization of Vm
ClC-2	Inward $Cl^->Br^->I^-$	Opens at - V_m	~3-5	9-AC, DPC	Swelling, Voltage	Volume Regulation
ClC-2G	Linear $I^->Cl^->NO_3^-$	Opens at + V_m	~7	?	PKA, pH	HCl secretion
ClC-3	Outward $I^->Cl^-=Br^-$	Voltage Independent	?	DIDS	PKC, CaCMK	Memory formation
ClC-5 [1]	Outward $I^->Cl^->F^-$	Opens at + V_m	?	DIDS, DPC	Voltage	Urine Formation
ClC-K1	Outward $Br^->Cl^->I^-$	Voltage Independent	?	DIDS, 9-AC	pH, Ca^{2+}	Urine Concentration
ClC-K2	Outward $Br^->Cl^->I^-$	Opens at all V_m	?	DIDS	Voltage	Urine Concentration

Table 1.3.2. Electrophysiological properties of the recombinant ClC Cl^- channels.

---

[1] There are two ClC-5 clones that were identified in rat kidney and rat brain. However, only the kideny ClC-5 cloned which expressed in CHO-K1 cells shows the listed properties, whereas the rat brain ClC-5 cloned shows $Cl^->I^-$ and lack of blockers sensitivity when expressed in *Xenopus* oocytes.

1.4 Cell volume regulation in epithelia

Volume regulation is crucial for all living cells to maintain proper cellular function. Cells act as perfect osmometers when exposed to an iso-osmotic environment. Cell shrinkage results from a net loss of water to the hypertonic medium, whereas cell swelling occurs when they are exposed to hypotonic medium, due to an influx of water. Osmotic stress is present even under normal physiological conditions. High concentrations of cytosolic impermeant, charged organic macromolecules create a constant driving force to pull small ions and water into the cell (Sarkadi & Parker 1991). If unregulated, cells would swell, eventually lyse and die. Cell shrinkage can be experienced by cells that are exposed to a wide range of osmolarities, such as the epithelia in the kidney and the intestine.

Cell volume is normally maintained by the actions of transporters or pumps that extrude or absorb solutes, followed by a corresponding movement of water to restore cell volume. Cell swelling stimulates effluxes whereas cell shrinkage enhances an influxes of solutes in order to achieve regulatory volume decrease (RVD) and regulatory volume increase (RVI), respectively (Sarkadi & Parker 1991). These defense mechanisms for volume changes allow cells to regulate their cell volume within minutes after osmotic perturbation.

Shrunken cells restore their volume by accumulating Na^+ and Cl^- via exchangers and/or cotransporters (Sarkadi & Parker 1991). An electrically silent 1:1 Na^+/H^+ exchanger, with concomitant activation of a Cl^-/HCO_3^- exchanger has been reported in variety of cells, including epithelial cells (Civan *et al.*, 1994; Hajjar *et al.*, 1995). Hypertonic stress activates the Na^+/H^+ exchanger, with an increase in cellular pH due to a net loss of H^+. Increased intracellular pH subsequently leads to elevation of HCO_3^- by carbonic anhydrase. The increase in HCO_3^- level then stimulates the influx of Cl^- via the Cl^-/HCO_3^- exchanger to accumulate intracellular NaCl. Other mechanisms, such as activation of the $Na^+/K^+/2Cl^-$ cotransporter for RVI have also been reported in many epithelia (Eveloff & Warnock 1987; MacLeod & Hamilton 1990; Ussing 1986). Moreover, a Na^+-coupled uptake of organic osmolytes for RVI was demonstrated in renal epithelia (Handler & Kwon 1993).

Most cells extrude cytosolic KCl and/or organic solutes during RVD in response to osmotic swelling (Sarkadi & Parker 1991). There are two general mechanisms for RVD, a transporter/exchanger pathway and a conductive pathway involving ion channels (Sarkadi & Parker 1991). Three different types of transporter/exchanger are involved in RVD, the electroneutral KCl cotransporter (Kennedy 1994), Na^+/Ca^{2+} exchanger (Shimizu *et al.*, 1992) and K^+/H^+ exchanger that couples with Cl^-/HCO_3^- exchangers (Reuss 1983). Activation of these transporters leads to a net loss of cellular osmolytes and cell volume can be restored by the corresponding water movement.

Swelling-activated K^+ and Cl^- channels have been reported in several different epithelial cells. Activation of these channels leads to a net loss of KCl and therefore returns cell volume to normal. However, it was recently reported that a swelling-activated Cl^- channel was also responsible for amino acid efflux during RVD (Strange & Jackson 1995). The biophysical properties of swelling-activated K^+ channels seem to be diverse. Swelling-activated K^+ channels were reported to be Ba^{2+}- and quinine-sensitive (Adorante & Cala 1995; Diener *et al.*, 1992; Banderali & Roy 1992). Single-channel conductances of swelling-activated K^+ channels have included 25 pS with linear current-voltage relationship (Banderali & Roy 1992), 30-56 pS with inward rectification (Nilius *et al.*, 1995a; Weiss & Lang 1992), and 150 pS "maxi" channels (Edelman *et al.*, 1995; Park *et al.*, 1994).

Swelling-activated Cl^- channels, like swelling-activated K^+ channels, also exhibit significant diversity. Three proteins molecules, the pI_{Cln}, ClC-2 and the human multidrug resistance (MDR1) gene product P-glycoprotein (Pgp), have been associated with swelling-activated Cl^- conductances (Gill *et al.*, 1992; Paulmichl *et al.*, 1992; Thiemann *et al.*, 1992; Valverde *et al.*, 1992).

pI_{Cln} was first identified from the Madin-Darby canine kidney cell line by an expression cloning method in *Xenopus* oocytes (Paulmichl *et al.*, 1992). When expressed in *Xenopus* oocytes, pI_{Cln} induced an outwardly rectifying Cl^- current (I_{cln}) upon hypo-osmotic challenge. I_{cln} showed an anion selectivity of $I^- > Cl^-$ and lack of

Ca^{2+}-dependence. Moreover, I_{cln} showed voltage- and time-dependent inactivation if the holding potential was greater than +40 mV. I_{cln} was also sensitive to blockade by the Cl^- channel blockers NPPB and DIDS, as well as extracellular nucleotides (Paulmichl *et al.*, 1992; Gschwentner *et al.*, 1995). Coinjection of antisense oligodeoxynucleotides and antibodies of pI_{Cln} into *Xenopus* oocytes caused a drastic reduction in I_{cln}, suggesting a direct involvement of pI_{Cln} in RVD (Krapivinsky *et al.*, 1994; Gschwentner *et al.*, 1995).

Support for pI_{Cln} function as a swelling-activated Cl^- channel came from mutation experiments. Mutation of a putative nucleotide-binding site on pI_{Cln} caused a loss of sensitivity to extracellular nucleotide and induced a dependence on extracellular Ca^{2+} (Paulmichl *et al.*, 1992). However, several studies have suggested a regulatory role for pI_{Cln} in RVD, rather than as a swelling-activated Cl^- channel. The first evidence suggesting a regulatory role for pI_{Cln} came from the original oocyte expression experiments (Paulmichl *et al.*, 1992). In spite of the common observation of a swelling-activated Cl^- current associated with the expression of pI_{Cln}, four percent of the uninjected oocytes also exhibited volume-activated current. Subsequent study demonstrated that a native swelling-activated Cl^- conductance was induced in over ninety percent of the uninjected oocytes (Ackerman *et al.*, 1994). Hydropathy analysis also failed to predict any transmembrane spanning segments for pI_{Cln}. However, it was proposed that a functional swelling-activated Cl^- channel could be formed by a pI_{Cln} dimer in the form of a ß barrel pore, instead of an α-helical structure (Paulmichl *et al.*, 1992). Furthermore, recent findings indicated that pI_{Cln} was most abundant in the cytosol, suggesting a regulatory role in volume regulation rather than a functional Cl^- channel in the plasma membrane (Krapivinsky *et al.*, 1994).

The second protein, ClC-2, that has been associated with RVD belongs to the ClC voltage-dependent Cl^- channel family (Thiemann *et al.*, 1992). ClC-2 was first isolated from a cDNA library of rat brain and heart using the cDNA encoding the rat skeletal muscle ClC-1 Cl^- channel (Thiemann *et al.*, 1992). The ClC-2 sequence encodes a 907 amino acid protein with 49% and 55% homology to the Torpedo ClC-0 and rat skeletal muscle ClC-1 Cl^- channels, respectively (Thiemann *et al.*, 1992). Like ClC-0 and ClC-1, ClC-2 consists of 12 hydrophobic, putatively transmembrane domains, with the activation domain located at the amino terminus (Gründer *et al.*, 1992). In contrast to the tissue-specific expression of ClC-0 and ClC-1, ClC-2 was ubiquitously expressed in many cell types, including fibroblasts, neuronal cells and epithelial cells (Thiemann *et al.*, 1992).

When ClC-2 was expressed in *Xenopus* oocytes, a swelling-activated Cl^- current was observed under hypotonic stress (Gründer *et al.*, 1992; Thiemann *et al.*, 1992). The ClC-2-induced Cl^- current also showed voltage-dependence, with increased channel activities at nonphysiological hyperpolarizing potentials (Gründer *et al.*, 1992; Thiemann *et al.*, 1992). This current activated slowly and showed slight inward rectification. Unlike pI_{Cln}, ClC-2 was more permeable to Cl^- than to I^- and showed no sensitivity to the Cl^- channel blockers NPPB and DIDS (Thiemann *et al.*, 1992). The single-channel conductance of the ClC-2 Cl^- channel was reported to be ~3-5 pS (Jentsch *et al.*, 1995).

P-glycoprotein, the product of the MDR1 gene, has been suggested to function both as a swelling-activated Cl^- channel and as a drug transporter (Gill *et al.*, 1992; Valverde *et al.*, 1992). Pgp belongs to the ATP-binding cassette superfamily of active transporters which utilize energy from ATP hydrolysis to sustain transport activity. It was shown that Pgp actively pumps cytotoxic drugs out of cells and therefore drug tolerance develops in some cell lines and human tumours (Gottesman & Pastan 1993).

When cells were transfected with the MDR1 cDNA encoding Pgp, an outwardly rectifying whole-cell Cl^- current was induced by hypotonicity (Díaz *et al.*, 1994; Gill *et al.*, 1992; Valverde *et al.*, 1992; Wu *et al.*, 1996). The Pgp-associated swelling-activated Cl^- current showed voltage-dependent inactivation at depolarizing potentials and time-dependent activation at hyperpolarizing potentials. However, intracellular ATP was essential for development of the Pgp-associated swelling-activated Cl^- current. The Pgp-associated swelling-activated Cl^- current showed sensitivity to the Cl^- channel blockers NPPB and DIDS, as well as to drug transport inhibitors for Pgp (Díaz *et al.*, 1994; Gill *et al.*, 1992; Valverde *et al.*, 1992; Wu *et al.*, 1996), suggesting a dual function for Pgp as a transporter as well as a Cl^-

channel. However, involvement of Pgp in RVD remains uncertain. Studies have shown that the swelling-activated Cl^- current was not directly related to the expression of Pgp (Rasola *et al.*, 1994; Tominaga *et al.*, 1995). Furthermore, other studies also failed to observe any inhibitory effects of inhibitors for transport on the Pgp-associated, swelling-activated Cl^- conductance (McEwan *et al.*, 1992; Tominaga *et al.*, 1995).

1.5 Purinergic receptors

Intracellular ATP plays an important role in energy metabolism and many other cellular regulatory functions. However, it is only comparatively recently that the effects of extracellular ATP and other nucleotides on cellular functions have been closely examined. Physiological effects of extracellular ATP on cardiac performance were first reported by Drury and Szent-Gyorgyi (1929) and subsequently on various other systems, including the nervous, inflammatory, and pulmonary systems (Dubyak & El-Moatassim 1993). Extracellular adenine nucleotides are now widely recognized as common regulators for many physiological functions. Extracellular ATP has been reported to increase Cl^- secretion in both normal and CF epithelia (Stutts *et al.*, 1994), and was suggested to have possible therapeutic potential in CF treatment (Knowles *et al.*, 1995).

Extracellular adenine nucleotides are released from intracellular sources via different mechanisms. Exocytotic release of granular ATP represents the major source of extracellular adenine nucleotides from both neuronal and non-neuronal cells. It has been shown that ATP is co-packaged and released with catecholamines from adrenal chromaffin granules (Castillo *et al.*, 1992), and synaptic vesicles (Von Kugelgen & Starke 1991). Co-release of ATP has also been observed with platelet dense granules (Meyers *et al.*, 1982) and with histamine from mast cells (Osipchuk & Cahalan 1992). Another pathway for the release of cytosolic nucleotides is via intrinsic plasma membrane proteins. It was shown that cytosolic ATP or adenosine were released from intact cardiac myocytes (Clemens & Forrester 1981) and erythrocytes (Forrester 1990) when they experienced hypoxia. Such release of ATP could be blocked by inhibitors of anion and nucleoside transporters. Other plasma membrane proteins such as P-glycoprotein and the cystic fibrosis transmembrane conductance regulator, members of the ABC transporter superfamily, have recently been reported to function as ATP-permeable channels (Abraham *et al.*, 1993, Reisin *et al.*, 1994, Schwiebert *et al.*, 1995). Finally, cytosolic ATP is also released by membrane rupture during cell death.

Once released, extracellular ATP is quickly metabolized by ecto-ATPase and ectonucleotidase to adenosine (Dubyak & El-Moatassim 1993). Extracellular adenosine can be eliminated by active uptake via membrane transporters (Cass 1995), simple diffusion back into the cells, or deamination by adenosine deaminase (Olsson & Pearson 1990). The sequential breakdown of ATP and reuptake of adenosine would then allow termination of the signal.

Extracellular adenosine or other adenine nucleotides mediate their effects via surface P_1 or P_2 purinergic receptors. The original classification (Burnstock 1978) of P_1 and P_2 receptors was based on their relative sensitivities to adenosine and ATP. P_1 receptors have a relative sensitivity of adenosine > AMP ≥ ADP ≥ ATP, whereas P_2 shows a reversed selectivity sequence. The P_1 receptor was recently re-classified into several subtypes, A_1, A_{2a}, A_{2b}, A_3 and possibly A_4 on the basis of selectivity for different adenosine analogs. The A_1 type has a selectivity sequence of (-)-N^6-(R-phenylisopropyl)adenosine (R-PIA) > 5'-*N*-ethylcarboxamidoadenosine (NECA) > (+)-N^6-phenylisopropyladenosine (S-PIA), whereas A_2 receptors show higher sensitivity to NECA than R-PIA (Dalziel & Westfall 1994; Olah & Stiles 1995). Subdivision of A_2 receptors into A_{2a} and A_{2b} is based on the differences in affinities to NECA, with A_{2a} showed higher affinity than A_{2b} (Olah & Stiles 1995). On the other hand, A_3 receptors have a selectivity sequence that can be classified as neither A_1 nor A_2, with R-PIA = NECA > S-PIA (Linden 1994). As for the putative A_4 receptor, NECA is not an effective agonist (Dalziel & Westfall 1994).

All adenosine receptors belong to the classic G-protein coupled receptor family (Dalziel & Westfall 1994; Olah & Stiles 1995), with the exception of the A_4 subtype (Dalziel & Westfall 1994). Like G-protein coupled receptors such as adrenergic or muscarinic receptors, adenosine receptors have seven α-helical transmembrane domains, with extracellular amino and intracellular carboxy terminals. Other common features in the G-protein coupled receptors such as an aspartate residue in the second transmembrane domain, and cysteine in extracellular regions are all present in adenosine receptors (Olah & Stiles 1995). However, all adenosine receptors are smaller in size (37-45 KDa) than the classic G-protein coupled receptors, which have an average molecular weight of ~70-80 KDa (Furlong *et al.*, 1992; Linden 1994; Ren & Stiles 1994).

Adenosine receptors show diverse G-protein-effector couplings. Inhibition of adenylyl cyclase and therefore, reduction in cellular cAMP upon receptor stimulation is typical for A_1 receptor activation (Gerber & Gahwiler 1994; Ma & Green 1992). A_1-induced reduction of cellular cAMP was reported to be mediated via pertussis-toxin sensitive G-proteins, suggesting that a G_o or G_i subtype of G-proteins was involved (Gerber & Gahwiler 1994; Ma & Green 1992). Recent evidence indicated that A_1 receptors could modulate PLC activities, also via a pertussis-toxin sensitive G-protein (Gerwins & Fredholm 1992).

Unlike A_1 receptors, A_{2a} predominantly activates adenylyl cyclase and increases cellular cAMP upon receptor stimulation (Chern et al. 1993; Liang & Haltiwanger 1995). Activation of adenylyl cyclase by A_{2a} was reported to couple the G_s subtype of the G-proteins (Chern et al. 1993). On the other hand, activation of A_{2b} receptor was demonstrated to increase PLC (Feoktistov & Biaggioni 1995; Yakel *et al.*, 1993), as well as adenylyl cyclase activities (Liang & Haltiwanger 1995; Strohmeier *et al.*, 1995). The A_3 receptor was shown to have similar multiple coupling to different G-proteins as the A_1 receptor. Activation of A_3 receptors causes an inhibition of adenylyl cyclase via coupling to a pertussis-sensitive G-protein (Zhou *et al.*, 1992) and increases intracellular Ca^{2+} by coupling to PLC pathways (Kohno *et al.*, 1996; Ramkumar *et al.*, 1993). The putative A_4 receptor is distinct from all the other adenosine receptors by its seeming lack of G-protein coupling, as well as its selectivity to various agonists (Olah & Stiles 1995).

P_2 receptors, like the P_1 receptors, are subdivided into five subtypes, P_{2X}, P_{2Z}, P_{2T}, P_{2Y}, and P_{2U} based on pharmacological properties (Dubyak & El-Moatassim 1993). These P_2 receptors are in general divided into two groups, the ATP-gated ion channel/pore (P_{2X} and P_{2Z}) and the G-protein coupled receptors (P_{2T}, P_{2Y} and P_{2U}). The P_{2X} receptor is a ligand-gated ion channel, with most abundant expression in neuronal and excitable tissue (Bean 1992; Fieber & Adams 1991). Activation of the P_{2X} receptor evokes an inward cationic current and membrane depolarization, with equal permeability to small ions like Na^+, K^+, Cs^+ and Ca^{2+} (Bean 1992). However, the majority of the P_{2X}-activated current is carried by Ca^{2+}, leading to an increase in cytosolic Ca^{2+} upon receptor activation (Bean 1992; Dubyak & El-Moatassim 1993). The single-channel conductance of the P_{2X} receptor ranges from 6 to 60 pS (Fieber & Adams 1991; Vincent 1992).

Two P_{2X} receptors have recently been cloned, with a selectivity sequence of 2-methylthio-ATP (2-MeSATP) ≥ ATP (Brake *et al.*, 1994; Valera *et al.*, 1994). However, these two P_{2X} clones differ from each other in their sensitivity to α,ß-methylene-ATP (AMP-C-PP) and degree of desensitization upon long exposure to agonists (Brake *et al.*, 1994; Valera *et al.*, 1994). The P_{2X} ATP-gated ion channels shows no homology to other ligand-gated channels, but rather show similarities to the inwardly rectifying K^+ channels, epithelial amiloride-sensitive Na^+ channels, and mechanosensitive channels from *Caenorhabditis elegans* (Brake *et al.*, 1994; Valera *et al.*, 1994).

P_{2Z} is another example of a non-G-protein coupled P_2 receptor with limited expression in immune cells such as lymphocytes, macrophages, and mast cells (Bretschneider *et al.*, 1995, Coutinho-Silva *et al.*, 1996, Naumov *et al.*, 1995, Tatham & Lindau 1990). Activation of P_{2Z} receptors causes an inward cationic current and permeabilization of plasma membrane to larger molecules such as Ba^{2+}, ethidium$^+$ and lucifer yellow via a non-selective pore. Permeabilization of plasma membrane by P_{2Z} receptor activation is mediated by the tetrabasic form of ATP, ATP^{4-}, and its effects on the membrane can be potentiated by reducing external divalent ions like Ca^{2+} and Mg^{2+}. P_{2Z}-induced pores have not been isolated, but their association with connexin has been demonstrated in mouse macrophages (Beyer & Steinberg 1991).

Signal transduction by P_{2Z}-induced pore formation is not yet understood.

Recent studies have indicated the involvement of the calmodulin-dependent pathways in ATP-induced cell lysis in macrophage and monocytic leukaemia cells (Blanchard *et al.*, 1995; Spranzi *et al.*, 1993). The single pore properties are also puzzling. It was predicted that in order to be permeable to molecules with molecular weights of 300-900 Da, the P_{2Z}-induced pore needed to have a size of ~200-400 pS (Tatham & Lindau 1990). However, electrophysiological recordings have not found P_{2Z}-associated channels of such conductance. Rather, the single-channel conductance has been reported to range from 2-17 pS (Bretschneider *et al.*, 1995, Coutinho-Silva *et al.*, 1996, Naumov *et al.*, 1995, Tatham & Lindau 1990). P_{2Z}-mediated separated channel and pore activities were recently reported in human macrophages, suggesting its dual effect on channel activation and pore formation (Blanchard *et al.*, 1995). The exact functions of P_{2Z} receptors in immunity and inflammation still remain unclear. It has been suggested that they might be involved in cell-mediated cytotoxicity (Di Virgilio 1995).

The P_{2T} receptor is a less well defined purinergic receptor, which shows both inotropic (Mahaut-Smith *et al.*, 1990) and G-protein coupled effects (Murgo *et al.*, 1994; Ohlmann *et al.*, 1995). Expression of P_{2T} receptors is restricted to platelets and their precursor cells (Murgo *et al.*, 1994; Soslau *et al.*, 1993). Activation of P_{2T} receptors causes platelets to undergo shape changes (from disc to spherical), aggregation and secretion of dense granules (Colman 1990; Soslau *et al.*, 1993). Unlike the other P_2 receptors, ADP is an agonist for the P_{2T} receptor, whereas ATP acts as an competitive antagonist (Colman 1990).

Mechanisms underlying the P_{2T}-induced platelet responses remain elusive. P_{2T} induces elevation of cellular Ca^{2+} which is well correlated with ADP-activated aggregation (Colman 1990). ADP-mediated increases in cytosolic Ca^{2+} were reported to result from influx of Ca^{2+} via a non-selective cation channel (Mahaut-Smith *et al.*, 1990), Ca^{2+} mobilization from intracellular stores via a PLC-dependent pathway (Daniel *et al.*, 1986; Murgo *et al.*, 1994), or both (Sage *et al.*, 1990). ADP-evoked Ca^{2+} mobilization was reported to be linked to a pertussis toxic-sensitive G-protein in erythroleukemia cells (Kesselring *et al.*, 1994). Besides the Ca^{2+} response, activation of P_{2T} receptors was also shown to inhibit adenylyl cyclase via coupling to G_i subtype of G-protein (Ohlmann *et al.*, 1995; Valeins *et al.*, 1992). However, inhibition of adenylyl cyclase was not associated with the ADP-induced aggregation (Mills *et al.*, 1985). Based on these observations, it has been suggested that ADP might activate two distinct P_{2T} receptors, one associated with ADP-gated cation channels, the other being a G-protein linked receptor.

Unlike the other P_2 receptors, P_{2Y} and P_{2U} receptors are widely expressed true G-protein coupled P_2 purinoceptors (Lustig *et al.*, 1993; Parr *et al.*, 1994; Webb *et al.*, 1993). Like the P_1 adenosine receptor, P_{2Y} and P_{2U} have a lower molecular weight (~42 KDa) and share many similarities to the classic G-protein coupled receptors. P_{2Y} and P_{2U} receptors show little homology to each other (~40%) (Parr *et al.*, 1994) and even less (< 30%) to other G protein-coupled receptors (Lustig *et al.*, 1993; Webb *et al.*, 1993).

P_{2Y} and P_{2U} receptors are two pharmacologically distinct purinoceptors (Lustig *et al.*, 1993; Parr *et al.*, 1994; Webb *et al.*, 1993). Both P_{2Y} and P_{2U} show affinity to ATP, however, P_{2Y} shows a relative potency order of 2-MeSATP > ADPßS > ATP >> AMP-C-PP = ß,γ-methylene-ATP, whereas P_{2U} shows no affinity to 2-MeSATP, ADPßS, or AMP-C-PP. In addition to ATP, P_{2U} receptors are also responsive to UTP, with a potency order of UTP ≥ ATP = ATPγS >> ADP. Both P_{2Y} and P_{2U} receptors have recently been cloned and therefore, support the existence of two individual receptors with distinct pharmacological profiles (Lustig *et al.*, 1993; Parr *et al.*, 1994; Webb *et al.*, 1993).

Activation of P_{2Y} and P_{2U} receptors, in general, causes an increase in cytosolic Ca^{2+} by mobilization from intracellular Ca^{2+} stores via a G-protein coupled, PLC-dependent pathway (Chen *et al.*, 1996; Cockcroft & Stutchfield 1989; Dubyak *et al.*, 1988; Siddiqui & Exton 1992; Zhang *et al.*, 1995). However, there are conflicting reports on the nature of such G-protein coupling. It was demonstrated that P_{2Y} or P_{2U} receptor-mediated PLC activities were coupled to a pertussis toxin-sensitive G-protein (Cockcroft & Stutchfield 1989; Zhang *et al.*, 1995), pertussis toxin-insensitive G-protein (Chen *et al.*, 1996; Siddiqui & Exton 1992) or both (Dubyak *et al.*, 1988).

In addition to their coupling to various G-proteins, P_{2Y} and P_{2U} receptors also show diverse coupling to cellular effectors. Increased cytosolic Ca^{2+} and PKC as a result of P_{2Y} or P_{2U} activation via PLC-dependent pathways were shown to give a secondary stimulation of phospholipase A_2 (PLA_2) and phospholipase D (PLD) (Balboa *et al.*, 1994; Lazarowski *et al.*, 1994; Pfeilschifter & Merriweather 1993). Activation of PLA_2 and PLD cause a release of archidonic acid (Lazarowski *et al.*, 1994) and accumulation of phosphatidic acid and 1,2-diacylglycerol, respectively (Balboa *et al.*, 1994; Pfeilschifter & Merriweather 1993). However, direct activation of PLA_2 by ATP, via a pertussis toxin-sensitive G-protein was also observed (Post *et al.*, 1996).

Other than their effects on overall Ca^{2+}-dependent cellular pathways, stimulation of P_{2Y} or P_{2U} receptors can also modulate adenylyl cyclase activity. ATP reduced cellular cAMP via a pertussis toxin-sensitive G-protein (Boyer *et al.*, 1993; Lin & Chuang 1993), but ATP-mediated cAMP accumulation was also observed through a direct coupling to adenylyl cyclase (Tada *et al.*, 1992) and an indirect pathway, which involves breakdown of archidonic acid, and the resulting metabolite prostaglandin, which then acts on its own receptor to increase cellular cAMP levels (Post *et al.*, 1996). The diverse coupling of purinergic receptors to PLC, PLA_2, PLD and adenylyl cyclase can therefore lead to modification of many cellular functions.

1.6 PLC-dependent inositol phosphates cycle

Cellular functions are regulated by many intracellular signalling molecules, some of which are generated by surface receptor activation. Activation of PLC-linked surface receptors, such as P_2 purinergic receptors, causes a rapid hydrolysis of membrane phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P_2) to generate two second messenger molecules, 1,2-diacylglycerol (DG) and Ins(1,4,5)P_3 (Berridge & Irvine 1989). Although PtdIns(4,5)P_2 is a preferred substrate for PLC, DG and Ins(1,4,5)P_3 can also be generated from hydrolysis of PtdIns*P* and PtdIns4*P*. Such changes in DG and Ins(1,4,5)P_3 and their subsequent effects on cellular Ca^{2+} and other enzymes are therefore responsible for regulating cellular functions such as metabolism, secretion, contraction, neural activity and cell proliferation (Berridge & Irvine 1989).

An increased level of DG enhances activation of protein kinase C (PKC) in the presence of two other cofactors, phospholipid (PL) and Ca^{2+} (Nishizuka 1988). Full activation of PKC requires binding of all three cofactors. However, there are at least seven species of PKC and activation of some of them is Ca^{2+}-independent (Nishizuka 1988). Besides PKC, a Ca^{2+}/calmodulin-dependent protein kinase (CaMK) can also be activated by PLC-receptor stimulation. Active PKC and CaMK then phosphorylate a wide array of cellular proteins and regulate a variety of cell functions.

Stimulation of PLC-linked receptors generates Ins(1,4,5)P_3 which in turn mobilizes Ca^{2+} from Ins(1,4,5)P_3-sensitive intracellular Ca^{2+} stores (Meldolesi *et al.*, 1990). The Ins(1,4,5)P_3-dependent intracellular Ca^{2+} stores are located in specialized endoplasmic reticulum (Meldolesi *et al.*, 1990) and its Ins(1,4,5)P_3-sensitivity results from expression of an Ins(1,4,5)P_3 receptor (InsP_3R) (Mignery *et al.*, 1989). Like the ryanodine receptor, InsP_3R is also an intracellular second messenger-gated Ca^{2+} channel (Jan & Jan 1992). Functional InsP_3R is formed as a tetrameric protein, with each subunit having a molecular weight of about 260 kDa (Mignery *et al.*, 1989). Each subunit consists of three domains: the Ins(1,4,5)P_3 binding domain in the amino terminal, a membrane spanning domain in the carboxy terminal and a long regulatory domain between the two terminals (Bezprozvanny & Ehrlich 1995). Overall, InsP_3R has six membrane-spanning regions with a relatively large intraluminal loop between the fifth and sixth transmembrane segments. Both the N- and C-terminals are located in cytoplasm and the large N-terminal forms a bulbous head that projects into the cytosol. A family of InsP_3Rs have been identified, with 60-70% homology to each other, and these are possibly formed by alternate splicing of a single gene (Bezprozvanny & Ehrlich 1995).

Binding of Ins(1,4,5)P_3 to InsP_3R causes a rapid release of Ca^{2+} into the cytosol. Four subconductance stages, each with a single-channel conductance of 20 pS have been observed upon Ins(1,4,5)P_3 binding to the receptor (Watras *et al.*, 1991). Intracellular Ca^{2+}, ATP and the phosphorylation state of InsP_3R were also shown to modulate the receptor and subsequent Ca^{2+} release (Bezprozvanny & Ehrlich 1995). A biphasic effect of cytoplasmic Ca^{2+} on InsP_3R was observed, with channel inhibition occurring when $[Ca^{2+}]_i$ was above 300 nM, whereas activation of the channel was facilitated by low $[Ca^{2+}]_i$ (Iino 1990). A potential Ca^{2+} binding site was identified on the regulatory domain of InsP_3R, and therefore, binding of Ca^{2+} on this site can directly modulate channel gating (Mignery *et al.*, 1992).

Intracellular ATP was shown to enhance Ins(1,4,5)P_3-gated Ca^{2+} efflux through InsP_3R (Ferris *et al.*, 1990; Maeda *et al.*, 1991). Channel potentiation by ATP did not involve phosphorylation of InsP_3R, rather, ATP enhanced Ca^{2+} efflux by allosteric modulation (Ferris *et al.*, 1990). Putative ATP binding sites have recently been located in the regulatory domain of InsP_3R. Finally, phosphorylation of InsP_3R was shown to have a modulatory effect on Ca^{2+} efflux (Burgess *et al.*, 1991; Ferris *et al.*, 1991; Volpe & Alderson-Lang 1990). Two PKA phosphorylation sites were identified on the regulatory domain (Maeda *et al.*, 1991) and phosphorylation of InsP_3R caused an isoform-dependent change in channel activity. Phosphorylation of InsP_3R reduced and enhanced its sensitivity to Ins(1,4,5)P_3 in neuronal (Volpe & Alderson-Lang 1990) and non-neuronal (Burgess *et al.*, 1991) isoforms, respectively. Other kinases such as PKC and CaMK were also demonstrated to modulate InsP_3R activity (Ferris *et al.*, 1991). Such phosphorylation by PKC and CaMK possibly occurs on sites that are distinct from the PKA sites. Phosphorylation of InsP_3R by PKA, PKC or CaMK then provides a cross-regulation by the adenylyl cyclase pathway and a feedback regulation by the PLC pathway on the overall Ca^{2+} signal.

Besides its function on the initial transient Ca^{2+} release from intracellular pools, Ins(1,4,5)P_3 was also shown to regulate Ca^{2+} influx upon receptor stimulation (Bird *et al.*, 1991; DeLisle *et al.*, 1995; Morris *et al.*, 1987). An Ins(1,4,5)P_3-gated Ca^{2+} channel has been reported in the plasma membrane of lymphocytes and olfactory cells (Kalinoski *et al.*, 1992; Khan *et al.*, 1992). However, these putative plasma membrane Ins(1,4,5)P_3-gated Ca^{2+} channels are less specific, with demonstrated affinity to Ins(1,3,4,5)P_3 (Kalinoski *et al.*, 1992). In addition to direct activation of plasma membrane Ca^{2+} channels, Ins(1,4,5)P_3 was proposed to regulate Ca^{2+} influx via an indirect pathway, as in the capacitative entry model (Putney 1986). In this model, emptying of intracellular Ins(1,4,5)P_3-sensitive Ca^{2+} pools itself stimulates Ca^{2+} entry by some unknown mechanisms, as demonstrated in lacrimal and mast cells (Bird *et al.*, 1991; Hoth & Penner 1992). The existence of Ca^{2+} influx factors, which are released from empty Ins(1,4,5)P_3-sensitive Ca^{2+} stores, has been proven by biochemical protein isolation (Randriamampita & Tsien 1993) and electrophysiological experiments (Parekh *et al.*, 1993). These Ca^{2+} influx factors might provide an explanation for the Ins(1,4,5)P_3- and Ins(1,3,4,5)P_4-independent Ca^{2+} influx observed in mast cells (Hoth & Penner 1992).

The focus of inositol phosphate research has been mainly on Ins(1,4,5)P_3 and its role in Ca^{2+} signalling pathway. However, the metabolic network of inositol phosphates is complex and many inositol phosphates are formed subsequent to receptor activation and Ins(1,4,5)P_3 generation (Fig. 1.6) (Shears 1996). The relative fluxes of each inositol phosphate in this metabolic network are controlled by well coordinated enzymatic reactions. Emerging evidence has suggested that inositol phosphates other than Ins(1,4,5)P_3 also have important physiological functions.

The cellular level of Ins(1,4,5)P_3 in a typical cell is generally about 0.1-0.2 μM, and increases to less than 1 μM upon stimulation within 5 s (Irvine *et al.*, 1985). Once generated, Ins(1,4,5)P_3 is quickly metabolized in *vivo* with a half-life of about 10 s (Irvine *et al.*, 1985). There are two metabolic pathways for the breakdown of Ins(1,4,5)P_3. It can be dephosphorylated to Ins(1,4)P_2 and eventually to inositol to replenish the inositol pool by various phosphatases (Balla *et al.*, 1988). The other metabolic pathway for Ins(1,4,5)P_3 is to be phosphorylated to Ins(1,3,4,5)P_4 by the Ins(1,4,5)P_3 3-kinase (Fig. 1.6) (Balla *et al.*, 1988). These enzymatic reactions then ensure the termination of the Ins(1,4,5)P_3 response.

Ins(1,3,4,5)P_4 has received great interest for its potential role as another second messenger, similar to Ins(1,4,5)P_3. It was found that Ins(1,3,4,5)P_4 showed affinity to InsP_3R and mobilized Ca^{2+} from Ins(1,4,5)P_3-sensitive pools (Delisle *et al.*, 1994; Irvine 1992; Parker & Ivorra 1991). However, Ins(1,3,4,5)P_4 was not as potent an agonist as Ins(1,4,5)P_3 in mobilizing Ca^{2+}, and the nature of the Ca^{2+} response was also different (Parker & Ivorra 1991). Unlike the rapid transient increase in cellular Ca^{2+} evoked by Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 was reported to induce only a delayed oscillating Ca^{2+} response. However, no Ca^{2+} mobilizing effect of Ins(1,3,4,5)P_4 was observed in other studies (Balla *et al.*, 1991; Bird & Putney 1996). In addition to its putative role in Ca^{2+} mobilization, Ins(1,3,4,5)P_4 was also shown to have conflicting effects on Ins(1,4,5)P_3-induced Ca^{2+} responses (Bird & Putney 1996; Parker & Ivorra 1991).

Similarly to Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 was also proposed to mediate Ca^{2+} influx either by itself (Hashii *et al.*, 1993; Smith 1992) or in combination with Ins(1,4,5)P_3 (Bird *et al.*, 1991; Morris *et al.*, 1987). An Ins(1,3,4,5)P_4-dependent Ca^{2+} channel has been identified on the plasma membrane of endothelial cells (Lückhoff & Clapham 1992). However, unlike the Ins(1,4,5)P_3-gated Ca^{2+} channel in lymphocyte and olfactory cells, the Ins(1,3,4,5)P_4-dependent Ca^{2+} channel was not sensitive to Ins(1,4,5)P_3, with an estimated conductance of 2.5 pS. Yet, lack of Ins(1,3,4,5)P_4-induced Ca^{2+} influx was reported in various studies (Balla *et al.*, 1991; Bird & Putney 1996; DeLisle *et al.*, 1995; Morris *et al.*, 1987).

The metabolic breakdown of Ins(1,3,4,5)P_4 is an initial step towards a complicated and less well known portion of the agonist-induced inositol phosphate turnover network (Fig. 1.6). The metabolic breakdown Ins(1,3,4,5)P_4 to Ins(1,3,4)P_3 by 5-phosphatase is not as rapid as the breakdown of Ins(1,4,5)P_3 to Ins(1,4)P_2 (Li *et al.*, 1992; Vajanaphanich *et al.*, 1994). The level of Ins(1,3,4,5)P_4 slowly returns to

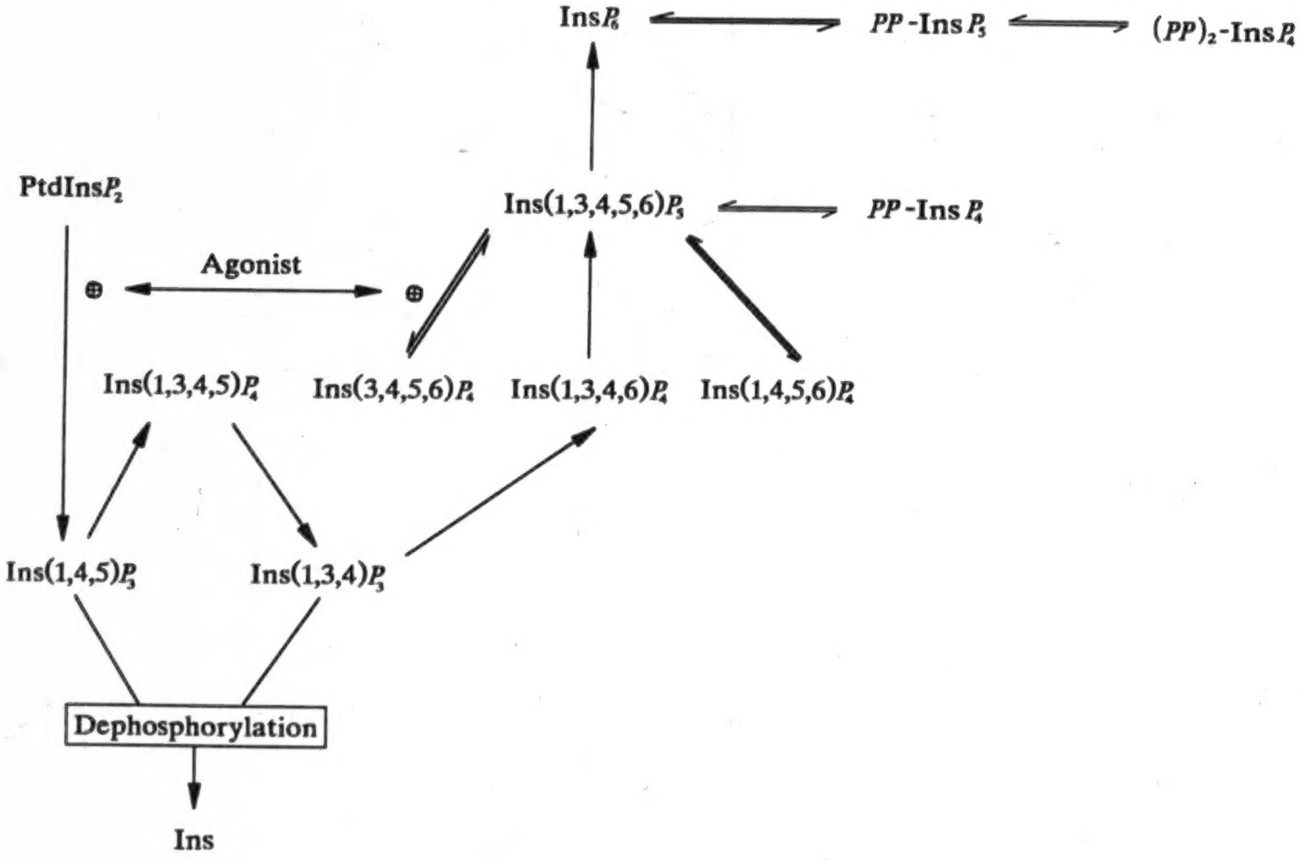
InsP_6
PP-InsP_5
$(PP)_2$-InsP_4
PtdInsP_2
Ins(1,3,4,5,6)P_5
PP-InsP_4
Agonist
⊕
⊕
Ins(1,3,4,5)P_4
Ins(3,4,5,6)P_4
Ins(1,3,4,6)P_4
Ins(1,4,5,6)P_4
Ins(1,4,5)P_3
Ins(1,3,4)P_3
Dephosphorylation
Ins

Figure 1.6. The generation of different inositol phosphates upon PLC-linked receptor activation by agonist in most animal calls (Shears 1996).

baseline over 3-5 min. In resting cells, the typical level of Ins(1,3,4)P_3 is about 1-4 μM and rises to 10-60 μM with stimulation (Shears 1996). The enzymatic pathway for dephosphorylation of Ins(1,3,4,5)P_4 back to Ins(1,4,5)P_3 by the Ins(1,3,4,5)P_4 3-phosphatase has already been demonstrated (Hughes & Shears 1990). However, it does not constitute a significant metabolic pathway for Ins(1,3,4,5)P_4. Unlike Ins(1,4,5)P_3, Ins(1,3,4)P_3 is unable to mobilize Ca^{2+} from the Ins(1,4,5)P_3-sensitive pools (DeLisle et al. 1994) although Ins(1,3,4)P_3 was reported to inhibit Ins(3,4,5,6)P_4 1-kinase activity and the formation of Ins(1,3,4,5,6)P_5 (Craxton *et al.*, 1994).

Ins(1,3,4)P_3 is a critical branch point in inositol phosphate metabolism. It can either be sequentially dephosphorylated by various specific phosphatase to inositol for resynthesis of inositol lipid, or be phosphorylated to Ins(1,3,4,6)P_4 by Ins(1,3,4)P_3 6-kinase and eventually, to higher charged inositol polyphosphates (Balla *et al.*, 1989; Hughes *et al.*, 1989) (Fig. 1.6). The Level of Ins(1,3,4,6)P_4 indicates a slow monophasic increase for as long as sixty minutes (Vajanaphanich *et al.*, 1994), suggesting prevalent Ins(1,3,4)P_3 6-kinase activity. In addition to the dephosphorylation and 6-kinase pathways, Ins(1,3,4)P_3 can also be rephosphorylated back to Ins(1,3,4,5)P_4 by 5-kinase (Hughes *et al.*, 1989). However, 5-kinase activity only represents a small fraction (~16%) of the total phosphorylation, and there is no evidence to indicate that such activity could sustain the measured level of Ins(1,3,4,5)P_4 (Hughes *et al.*, 1989).

Functionally, Ins(1,3,4,6)P_4 is very similar to Ins(1,3,4,5)P_4 in its action on the Ca^{2+} signal. Ins(1,3,4,6)P_4 mobilized Ca^{2+} from the Ins(1,4,5)P_3-sensitive stores (DeLisle *et al.*, 1994; Ivorra *et al.*, 1991), induced Ca^{2+} influx (DeLisle *et al.*, 1995; Ivorra *et al.*, 1991) and enhanced the Ins(1,4,5)P_3-induced Ca^{2+} response (Ivorra *et al.*, 1991). In general, Ins(1,3,4,6)P_4 is several times more potent in inducing a Ca^{2+} signal than Ins(1,3,4,5)P_4 (DeLisle *et al.*, 1994; Ivorra *et al.*, 1991). However, short term regulation of the Ca^{2+} signal by Ins(1,3,4,6)P_4 is questionable due to its prolonged formation. Instead, Ins(1,3,4,6)P_4 might provide a longer term regulation of cellular function by maintaining the Ca^{2+} signal.

Ins(1,3,4,6)P_4 is further phosphorylated to Ins(1,3,4,5,6)P_5 by the Ins(1,3,4,6)P_4 5-kinase (Fig. 1.6) (Balla *et al.*, 1989; Hughes *et al.*, 1989). Although other InsP_5s like Ins(1,2,3,4,6)P_5, Ins(1,2,4,5,6)P_5 and Ins(2,3,4,5,6)P_5 also exist in animal cells, Ins(1,3,4,5,6)P_5 represents over 95% of the total InsP_5 (Shears 1996). The sequential phosphorylation from Ins(1,3,4)P_3 to Ins(1,3,4,5,6)P_5 is a relative slow process. It was reported to require up to 2-3 days before reaching the equilibrium level of InsP_5 (Hughes *et al.*, 1989). Even slower is the phosphorylation of Ins(1,3,4,5,6)P_5 to InsP_6 by Ins(1,3,4,5,6)P_5 2-kinase (Ji *et al.*, 1989). Nevertheless, the levels of Ins(1,3,4,5,6)P_5 and InsP_6 comprise a majority of the inositol phosphate content in mammalian cells and increase up to 60 μM upon stimulation (Sasakawa *et al.*, 1995).

The sluggish metabolic turnover of Ins(1,3,4,5,6)P_5 and InsP_6 suggests a lack of acute regulation of cellular functions. In regard to the overall Ca^{2+}, Ins(1,3,4,5,6)P_5 was reported to act as an antagonist for InsP_3R and therefore, inhibited Ca^{2+} mobilization (Lu *et al.*, 1996). In mammalian cells, Ins(1,3,4,5,6)P_5 and InsP_6 exhibit diverse regulatory functions other than contributing to the overall Ca^{2+} signal. First, InsP_6 was shown to have high affinity for a group of proteins including AP-2, AP-3, coatomer and arrestine (Palczewski *et al.*, 1992; Sasakawa *et al.*, 1995; Shears 1996). All of these proteins were reported to participate in either the control of vesicle traffic or receptor desensitization. Like InsP_6, Ins(1,3,4,5,6)P_5 also shows affinity to these proteins but to a lesser degree for coatomer (Shears 1996). It is therefore likely that Ins(1,3,4,5,6)P_5 and InsP_6 regulate receptor response by controlling the desensitization and internalization process. Second, Ins(1,3,4,5,6)P_5 and InsP_6 were demonstrated to have regulatory effects on ion channels (Sawada *et al.*, 1989a & 1989b; Vallejo *et al.*, 1987). When injected into *Aplysia Kurodai*, Ins(1,3,4,5,6)P_5 induced a slow, nonspecific cationic conductance, whereas InsP_6 induced a biphasic current, with initial Na^+ and Ca^{2+} inward currents and outward K^+ current (Sawada *et al.*, 1989a & 1989b). When applied extracellularly in the brainstem region, Ins(1,3,4,5,6)P_5 and InsP_6 were shown to reduce heart rate and blood pressure (Vallejo *et al.*, 1987). Finally, Ins(1,3,4,5,6)P_5 and InsP_6 exhibited high affinity binding to divalent and trivalent ions like Ca^{2+}, Mg^{2+}, Fe^{3+} and Zn^{3+} (Brown 1969). It has been proposed that InsP_6 may act as a physiological "iron chaperone" to transport ionized iron from carrier proteins across membrane barriers to the target site of utilization such as mitochondria (Hawkins *et al.*, 1993).

It is certain that InsP_6 does not represent a metabolic end point. Emerging evidence has suggested that InsP_6 can be further phosphorylated to form a new class of inositol phosphate, the inositol pyrophosphates (Shears 1996). The functions of these pyrophosphates are yet to be determined. The dephosphorylation of InsP_6 was shown to be minor (Nogimori *et al.*, 1991), whereas dephosphorylation of Ins(1,3,4,5,6)P_5 yields both Ins(1,4,5,6)P_4 and Ins(3,4,5,6)P_4 to form two "close-loop" substrate cycles (Menniti *et al.*, 1990; Oliver *et al.*, 1992). Yet, formation of Ins(1,3,4,6)P_4 was reported to be insignificant (Fig. 1.6) (Balla *et al.*, 1994).

Ins(1,4,5,6)P_4 is formed by dephosphorylation of Ins(1,3,4,5,6)P_5 by a multiple inositol polyphosphate phosphatase (MIPP), which also has affinity for InsP_6, several InsP_5s and InsP_3s (Nogimori *et al.*, 1991). Ins(1,4,5,6)P_4 can be rephosphorylated back to Ins(1,3,4,5,6)P_5 by a 3-kinase which like MIPP, shows affinity to other substrates such as Ins(1,2,4,5,6)P_5 (Oliver *et al.*, 1992). In contrast to all the inositol phosphates that have been discussed, no significant change of Ins(1,4,5,6)P_4 has been observed upon stimulation (Vajanaphanich *et al.*, 1994). Functionally, it was demonstrated that Ins(1,4,5,6)P_4 was not a potent agonist for InsP_3R despite its structural similarities to Ins(1,4,5)P_3 (DeLisle *et al.*, 1994). However, both Ins(1,4,5,6)P_4 and Ins(1,4,5)P_3 were reported to show high affinity to a 130 kDa protein (p130) (Kanematsu *et al.*, 1996). This p130 shows homology to the δ-isoform of PLC but expresses no PLC activity. The Ins(1,4,5,6)P_4/Ins(1,4,5)P_3-binding site of p130 is within the pleckstrin homology (PH) domain (Kanematsu *et al.*, 1996), which has been proposed to promote protein-protein interactions (Gibson *et al.*, 1994). It has been suggested that binding of Ins(1,4,5,6)P_4 to the PH domain might therefore modulate the transfer of proteins between the plasma membrane and the cytosol (Kanematsu *et al.*, 1996).

Another byproduct from dephosphorylation of Ins(1,3,4,5,6)P_5 is Ins(3,4,5,6)P_4, which is similar to Ins(1,4,5,6)P_4, and represents another metabolic "dead end" of inositol phosphate turnover (Menniti *et al.*, 1990; Oliver *et al.*, 1992) (Fig. 1.6). Unlike the transient increase in Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4, the level of Ins(3,4,5,6)P_4 was shown to increase slowly for up to 10 μM during prolonged stimulation (Vajanaphanich *et al.*, 1994), which corresponded to a parallel small decrease in Ins(1,3,4,5,6)P_5 (Menniti *et al.*, 1990). The Ins(1,3,4,5,6)P_5/Ins(3,4,5,6)P_4 cycle was reported to be controlled by two enzymes, Ins(1,3,4,5,6)P_5 1-phosphatase and Ins(3,4,5,6)P_4 1-kinase (Menniti *et al.*, 1990). Increase of Ins(3,4,5,6)P_4 upon stimulation has been proposed as the direct result of receptor-mediated increased activity of Ins(1,3,4,5,6)P_5 1-phosphatase (Menniti *et al.*, 1990). Activity of the Ins(1,3,4,5,6)P_5 1-phosphatase is independent of Ca^{2+} or PKC regulation (Menniti *et al.*, 1990), but requires the presence of ATP (Oliver *et al.*, 1992). Moreover, it was shown that Ins(3,4,5,6)P_4 1-kinase was inhibited by Ins(1,3,4)P_3 and therefore, enhanced the dephosphorylation of Ins(1,3,4,5,6)P_5 to Ins(3,4,5,6)P_4 upon stimulation (Craxton *et al.*, 1994). Receptor-mediated breakdown of Ins(1,3,4,5,6)P_5 to Ins(3,4,5,6)P_4 might signify the physiological importance of this inositol tetrakisphosphate.

As mentioned earlier, Ins(3,4,5,6)P_4 is a potent inhibitor of Ins(1,3,4)P_3 6-kinase, and therefore, imposes a restraint on the metabolic flux of Ins(1,3,4,6)P_4 and higher inositol polyphosphates (Hughes *et al.*, 1989). There has been little support for any Ins(3,4,5,6)P_4-mediated Ca^{2+} release from Ins(1,4,5)P_3-sensitive pools or Ca^{2+} influx (DeLisle *et al.*, 1994 & 1995; Lu *et al.*, 1996). However, there is emerging evidence that Ins(3,4,5,6)P_4 plays an important role in Cl^- secretion in epithelial cells. It was first reported in T84 cells that reduced Ca^{2+}-dependent short circuit Cl^- current by a second agonist challenge was correlated with the increase in cellular InsP_4s (Kachintorn *et al.*, 1993). It was subsequently shown that the level of Ins(3,4,5,6)P_4 was most closely related to a reduction of Cl^- secretion (Vajanaphanich *et al.*, 1994). The role of PKC in mediating such inhibition by receptor desensitization and/or down regulation was excluded and further supported an inhibitory role for Ins(3,4,5,6)P_4 (Traynor-Kaplan *et al.*, 1994). It is therefore crucial to understand the actions of Ins(3,4,5,6)P_4 on ion transport in epithelial cells and its implication in CF therapy. Details of the Ins(3,4,5,6)P_4 regulation of Cl^- current at the single cell level will be demonstrated and fully discussed in the Results and the Discussion sections.

1.7 Tissue culture in biological research

The study of ion transport in epithelia has been significantly enhanced by the use of tissue culture. Primary culture involves isolation of epithelial cells from target organs or tissues and maintenance in growth medium before use. In general, primary culture provides a short-term supply of cells, because epithelial cells eventually dedifferentiate and proliferation ceases within few passages. The origin of tissue for primary culture is also important. Considerations of differences between species is needed for the use of primary cultures of epithelia cells isolated from animals. Primary culture of human epithelia cells is often the best option. However, tissue availability can be a problem and ethical issues have to be addressed. Moreover, epithelial cells isolated from human subjects are quite variable, depending on the sex, age, state of health, and life style of the donor. Such differences in cell conditions can contribute to variable results.

The use of cultured cell lines has several advantages for studies of ion transport. Similarly to primary culture, cultured epithelial cell lines are isolated from various sources. However, these cultured epithelial cells have undergone transformation and become "immortal" with an increased growth rate (Freshney 1992). Transformation can occur naturally or by transfecting the cells with oncogenes. Some cell lines are derived from tumours and immortalization is an inherent characteristic of the tumour cells. Nonetheless, cultured epithelial cell lines may retain many characteristics that resemble the native cells, such as receptors for different hormones, cell shape and polarity. Cultured epithelial cells can be stable over many passages, providing a constant and convenient cell supply for transport research.

Besides their convenience, cultured cell lines also provide epithelial cells with homogenous populations for transport studies. In a native epithelium such as the lung, there are several different cell types, such as type I and II pneumocytes, Clara and globet cells. Each of these cells may have distinct transport properties. Moreover, The ability of cultured epithelial cell lines to grow as monolayers allows access to receptors expressed in the basolateral membrane. In native tissues, the basement membrane and lamina propria form a physical barrier and contain other elements that can alter epithelial function. Thus, cultured epithelial cells provide a purified membrane for transport studies without the influence of other components.

Culture conditions are crucial for transport studies with epithelial cell lines. Most lines are maintained in a defined medium, with added serum (mostly fetal calf or bovine serum) to produce a satisfactory growth rate. Complications may arise because the exact composition of the serum is unknown and likely to vary from batch to batch. Some of these unknown components may have different effects on protein expression, and therefore, variation in normal cell functions might occur. In spite of this problem, cultured epithelial cell lines can still provide useful models for ion transport research.

1.8 Electrophysiological techniques

The patch clamp technique (Neher & Sakmann 1976) has been widely used in the study of ion channels in variety of cells. There are four basic configurations, and each of them allows ion channels to be studied under different conditions. The on-cell or cell-attached mode permits direct recording of single-channels without the washout of important intracellular molecules. At the other extreme, the inside-out and outside-out modes study ion channels in isolated membrane patches. These recordings are done under well defined conditions, and allow manipulation of either the intra- or extra-cellular solutions. Finally, the whole-cell mode enables the study of a population of channels in the form of macroscopic currents, with some degree of washout of intracellular molecules. The patch clamp technique can provides direct measures of single-channel properties, including the kinetics of channel gating. However, the inherent noise in the recording places a limit on the minimum size of channel currents that can be observed.

Fluctuation or noise analysis provides an alternative technique for studying ion channels. Membrane current noise is created by any fluctuations of current around the mean current under voltage-clamp conditions. This membrane noise is principally made up of Lorentzian noise and background or 1/f noise (Van Driessche and Van Deynse 1990). Lorentzian noise results from random opening and closing of ion channels. The sum of these random fluctuations creates the total Lorentzian noise. The sources of 1/f noise are not well understood but probably include noise generated at the electrodes. This macroscopic current noise can give reliable microscopic information about the single-channel conductance, gating behaviour, channel density and open probability (Van Driessche & Zeiske 1985). Careful experimental design is needed to minimize errors arising from sources including the recording pipet and filtering system to allow accurate estimation of the microscopic channel properties.

1.9 Outline of the thesis

In this thesis I used the whole-cell patch clamp technique to study the regulation of swelling-activated and Ca^{2+}-activated Cl^- conductances in two different epithelial cell lines. Fluorescence measurements were also used to identify the possible purinergic receptor in one group of cells. The thesis is divided into three parts. In the first part, I used whole-cell patch clamp to characterize the swelling-activated Cl^- current in T84 cells. The results of this study are presented in Section 3.1. I then used noise analysis of the swelling-activated whole-cell current measured at a fixed holding potential to estimate the single-channel properties of this Cl^- channel. The results of the noise analysis experiments are presented in Section 3.2.

In the second part of this thesis, I identified a purinergic receptor in CFPAC-1 cells using fluorescence measurements of intracellular calcium concentration. These results are described in Section 3.3. In the final part of the thesis, I studied the effects of the purinergic receptor-mediated, Ca^{2+}-dependent Cl^- conductance in CFPAC-1 cells using the whole-cell patch clamp technique. The results from these experiments are described in Section 3.4. I then examined the regulation of this Cl^- conductance by various inositol phosphates, and these results are presented in Section 3.5. All of the results and their significance are discussed in Chapter 4.

2. MATERIALS AND METHODS

2.1 Cell culture

T84 and CFPAC-1 cells were obtained from the American Type Culture Collection (Rockville, MD), and were maintained in Dulbecco's modified Eagle's medium with Ham's nutrient mixture F-12 (DMEM-Ham F12) and Iscove's Modified Dulbecco's medium (IMDM), respectively. Each medium was buffered with $NaHCO_3$, at a concentration of 7.5% W/V for DMEM-Ham F12 and 3.02% W/V for IMDM. Both growth media were supplemented with the antibiotics penicillin (100 U/ml), streptomycin (75 U/ml) and gentamycin (0.5 mg/ml). To maintain a reasonable growth rate, both media were supplemented with fetal bovine serum, at a concentration of 5% in DMEM-Ham F12 and 10% in IMDM. The cultures were kept at 37°C in a humidified 5% CO2-95% air incubator and the medium was changed every two days.

For electrophysiological recording, confluent monolayers were subcultured by treatment with 0.05% W/V trypsin-0.02% W/V EDTA in Ca^{2+}- and Mg^{2+}-free Hank's buffered salt solution. Cells were then washed with the growth medium to remove trypsin residues and were recollected by centrifuging the cell suspension at 200 g for five minutes. Cells were resuspended in the growth medium at a plating density of $1\text{-}2\times10^4$ cells/ml. Cells were plated in 35 mm petri dishes (Falcon, Franklin Lakes, NJ) and were used within forty-eight and twenty-four hours after seeding for T84 and CFPAC-1 cells, respectively. The cells used for these studies were from passages sixty-five to eighty-five for T84 cells and passages twenty-two to thirty-two for CFPAC-1 cells. All culture media and trypsin solutions were purchased from Sigma Chemical Company (St. Louis, MO).

For the intracellular calcium measurement experiments, CFPAC-1 cells were grown on 25 mm circular microscope cover glass slips (Fisher Scientific, Ottawa, ON). Cover slips were first cleaned in a solution containing 2.1% H_2SO_4 and 95% ethanol for twenty minutes to remove any hydrocarbon coating. They were rinsed under tap water for ten minutes and then distilled water to remove any H_2SO_4 or ethanol residues. Cover slips were dried before sterilization. Individual sterile cover slips were then placed in 35 mm petri dishes for plating.

Similar trypsinization was used for both the calcium measurement experiments and the electrophysiological studies, except for the plating procedure. CFPAC-1 cells collected after trypsinization and washing were resuspended in the growth medium at a density of about 2-3x10^6 cells/ml. This high density cell suspension (0.5 ml) was directly added to the cover glass and kept in an incubator for one to two hours to ensure high density attachment on the cover glass before applying additional (1.5 ml) growth medium to cover the whole dish. Cells were used after two days and reached at least 80% confluence.

2.2 Electrophysiological recording

Conventional whole-cell patch clamp recording was used to measure all currents. Experiments were performed on single cells using pipets made from thin-walled (1.5 mm OD and 1.12 mm ID) borosilicate glass (A-M Systems Inc, Everett, WA) using a two-stage vertical puller (Nirashige, Japan). Pipet tips were fire polished to 2-5 MΩ immediately before experiments. Cells were washed three times with the bath solution before experiments. The detailed compositions of all solutions are listed in Table 2.2.1 & 2.2.2. Recordings were made with a List EPC-7 amplifier (Adams & List, Germany). For measuring current-voltage relationships the voltage was controlled and the current data were gathered by an IBM compatible computer, using 12-bit digital-to-analog and analog-to-digital convertors. The holding potential was -40 mV and the membrane potential was first stepped to -100 mV for 50 ms to remove any voltage-dependent inactivation of ion channels. Cells were then stepped to voltages in the range -90 mV to +90 mV with increments of 20 mV for a period of 450 ms. For current-voltage relationships, the steady-state current was obtained from the mean current measured during the final 10 ms at each potential.

Different experimental conditions were used to produce specific whole-cell currents. The swelling-activated current was induced by imposing a transmembrane osmotic gradient and the current was measured every minute after formation of the whole-cell configuration. Ca^{2+}-dependent currents were induced by adding 2 μM ATP or 1 μM thapsigargin (TG) (Sigma Chemical Company, St. Louis, MO) to the bath solution. For studies of the regulation of Ca^{2+}-dependent current, inositol phosphates were obtained from several sources: Ins(1,3,4,5)P_4 was obtained from Calbiochem, La Jolla, CA; Ins(1,3,4,5,6)P_5 was purchased from Sigma Chemical Company, St. Louis, MO; Ins(1,4,5,6)P_4 and Ins(3,4,5,6)P_4 were synthesized by Dr. K.S. Bruzik as described previously (Xie *et al.*, 1996); and Ins(1,3,4,6)P_4 was a kind gift from Dr. R. Gigg, National Institute for Medical Research, Mill Hill, London, UK. Inositol phosphates were dissolved in the pipet solution (Table 2.2.2) before the experiment and the cell interior was allowed to equilibrate with the pipet solution for two minutes prior to recording, to ensure proper mixing of the pipet solution with the cytosol. The dose-response curve for the reduction of Ca^{2+}-dependent current by Ins(3,4,5,6)P_4 was fitted by:

$$I=\frac{I_{max}}{1+(c/K)^{a}}+I_{base} \qquad (1)$$

where I_{max} is the maximum current blocked by Ins(3,4,5,6)P_4, I_{base} is the current unaffected by Ins(3,4,5,6)P_4, c is the concentration of Ins(3,4,5,6)P_4, K is an apparent equilibrium constant for the reaction between Ins(3,4,5,6)P_4 and its intracellular target, and a is the cooperativity factor (Copello *et al.*, 1991).

Experiment/ Chemical (mM)	NaCl	KCl	$CaCl_2$	$MgCl_2$	HEPES	EGTA	SUCROSE	pH
Swelling: Normal	140	5	2	1	10	0	0	7.3
Swelling: Hypertonic	140	5	2	1	10	0	20	7.3
Ca^{2+}: Normal	140	5	2	1	10	0	0	7.4
Ca^{2+}: Zero-Ca^{2+}	140	5	0	1	10	2	3	7.4
$[Ca^{2+}]$i measurement: Noraml	145	5	2	2	10	0	10	7.4
$[Ca^{2+}]$i measurement: Zero-Ca^{2+}	145	5	0	2	10	0	10	7.4

Table 2.2.1. Compositions of different bath solutions used in all experiments.

Experiment/ Chemical (mM)	NaCl	KCl	$CaCl_2$	$MgCl_2$	HEPES	EGTA	SUCROSE	pH
Volume: Normal	5	140	0.5	2	10	5	0	7.3
Volume: Hypertonic	5	140	0.5	2	10	5	20	7.3
Ca^{2+}: Normal	5	140	0.19	2	10	0.5	0	7.4
Ca^{2+}: Zero-Ca^{2+}	5	140	0	2	10	5	0	7.4

Table 2.2.2. Compositions of different pipet solutions used in all whole-cell patch-clamp experiments.

The relative contribution of Cl^- current to total whole-cell current was determined by the reversal potential of the whole-cell current and by the sensitivity of whole-cell current to the Cl^- channel blocker 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) (Sigma Chemical Company, St. Louis, MO). DIDS was dissolved in the bath solution before experiment. The reversal potential for each ion was calculated from the Nernst equation (Hille 1992):

$$E_x = \frac{RT}{ZF} \ln \frac{[x]_o}{[x]_i} \tag{2}$$

where R is the gas constant, T is the absolute temperature at 20°C, F is the Faraday's constant, Z is the valency, $[x]_o$ and $[x]_i$ are the extra- and intracellular concentrations for ions.

2.3 Intracellular calcium measurements

Intracellular calcium measurements were performed on cells prepared as described in the Section 2.1, using the fluorescent calcium indicator Fura-2/AM (Molecular Probes, Eugene, OR). Fura-2/AM was dissolved in dry dimethyl sulfoxide and was subsequently diluted in culture medium to give a final loading concentration of 2 μM. Cell loading was then performed in the dark at 20°C-25°C for 60 minutes. Before $[Ca^{2+}]_i$ measurements, cells were washed three times with bath solution (Table 2.2.1) and the cover slip was mounted onto a Sykes-Moore chamber with 1 ml of bath solution. The chamber was then placed on an inverted epifluorescence microscope (Nikon, Japan) for recording. Cells were allowed to remain in the bath solution for five minutes before proceeding with the experiments.

The ultraviolet (UV) light source was a dual-excitation spectrofluromettric system (Spex Industries, Edison, NJ), with the excitation wavelengths adjusted to 340 nm and 380 nm by filters. The excitation UV light was then directed to the cell layer by a dichroic mirror with a cut-off wavelength of 400 nm. A 40X phase-contrast objective lens (Nikon, Japan) was used to transmit and condense the excitation UV light towards the cell layer and also to collect and to focus the fluorescence emitted from the Fura-2 loaded cells. The total fluorescence signal (above 400 nm) was then directed to a photomultiplier. The amplified signal was sampled by a computer coupled to a spectrofluorometer using the CM3000DM program (Spex Industries, Edison, NJ). The signal was sampled alternately between the two excitation wavelengths of 340 nm and 380 nm every second, with an integration time of 300 ms. A simultaneous real-time plot of fluorescence intensity for both excitation wavelengths was generated and stored in a computer. The change in $[Ca^{2+}]_i$ was expressed as the ratio of fluorescence intensity measured with excitation wavelengths of 340 nm and 380 nm for each pair of data points.

Extracellular ATP and UTP were used to stimulate Ca^{2+} response in CFPAC-1 cells. Agonists were added to the recording chamber after a stable fluorescence baseline had been established, about 100 s after the start of the experiment. Intracellular Ca^{2+} was then monitored for 300 s after application of the agonists. Control experiments were performed by injection of equal amounts of water into the recording chamber. To determine the source(s) of the agonist-induced Ca^{2+} signal, Ca^{2+} in the bath solution (Table 2.2.1) was removed or the cells pre-incubated with 70 nM TG for ten minutes to deplete intracellular Ca^{2+} stores.

2.4 Noise analysis

Noise analysis was used to estimate the properties of the Cl^- channels causing the swelling-activated current. The development of the swelling-activated Cl^- current was measured at a constant holding potential. For continuous recordings of membrane current versus time, the voltage was controlled by a constant voltage source and the current recording was stored in digital format on videotape using a 16-bit analog to digital convertor. The bandwidth of the recording system was 0-20 kHz. For membrane current noise analysis, whole-cell currents were low-pass filtered by a 9-pole, 300 Hz, active analog filter. The current was sampled at 1 ms intervals and digitized by a 12-bit analog-to-digital convertor for storage on a computer disk. Data was processed in segments of 4,096 sample points. To remove the current trend, each segment was first fitted by a second-order polynomial in time, using linear regression. The fitted polynomial was then subtracted from the original data. Total variance was calculated from the mean of the squared residual current values. For Lorentzian fitting, each segment was processed by the fast Fourier transform (Cooley *et al.*, 1970) and spectra from a series of segments were averaged. Spectra were fitted by a double Lorentzian function of the form:

$$\sigma^2 = \frac{S_1}{1+(f/\theta_1)^2} + \frac{S_2}{1+(f/\theta_2)^2} \tag{3}$$

where σ^2 is the total variance, S_1, S_2 are the low frequency plateaux, θ_1, θ_2 the corner frequencies of the two Lorentzian functions, and f is frequency (Lindemann & Van Driessche, 1977). Fitting was performed by the Levenberg-Marquardt general nonlinear fitting algorithm (Press *et al.*, 1990) over the range 0-180 Hz, where the filter output was 99-100% of the input. The variance of each Lorentzian component was obtained by integrating over frequency.

The single-channel conductance of the time dependent current, γ, was obtained by fitting the noise variance versus membrane current relationship with:

$$\sigma^2 = \sigma_0^2 + I(V-E)\gamma - I^2/N \tag{4}$$

where σ_0^2 is the background variance, I is the total membrane current, V is the membrane potential, E is the reversal potential, and N is the number of channels (Ehrenstein *et al.*, 1970; Sigworth 1980). This second-order polynomial in membrane current was fitted by linear regression, with the linear term giving an estimate of γ

and the quadratic term an estimate of N. The open probability of the channels, P_o, was then calculated as a function of membrane current, I:

$$P_o = I/[N(V-E)\gamma] \tag{5}$$

2.5 Statistical Analysis

The statistical significance of the effects of ATP and UTP on $[Ca^{2+}]_i$ and swelling, and the effects of different inositol phosphates on whole-cell current were measured by unpaired, two-tailed T-tests.

3. RESULTS

3.1 Ionic basis of the swelling-regulated conductance in T84 cells [2]

3.1.1 Introduction

Volume regulation is essential for protecting living cells from membrane damage during osmotic changes. Acute volume changes can be compensated by temporarily changing the membrane conductance to small electrolytes or amino acids, so that the corresponding water movement restores cell volume. Swelling-activated ionic currents have been observed in a variety of epithelial cells (Diener *et al.*, 1992; Kubo & Okada 1992; Nilius *et al.*, 1994 & 1995b; Worrell *et al.*, 1989). A common mechanism for regulatory volume decrease (RVD) is by co-activation of both K^+ and Cl^- currents, but currents that are exclusively anionic have been reported in some cells (Grinstein *et al.*, 1983; Kelly *et al.*, 1994).

The regulatory mechanisms for swelling-dependent channels are diverse (Sarkadi & Parker, 1991) and a clear separation of swelling effects from other intracellular signals has not always been possible. Epithelial cells contain a variety of chloride channels and these can be affected by cyclic AMP, calcium and membrane potential, as well as swelling (Anderson *et al.*, 1992). Cross-sensitivity to swelling and voltage has been reported in several cases, with depolarization causing inactivation (Gill *et al.*, 1992; Kubo & Okada 1992; McCann *et al.*, 1989; Paulmichl *et al.*, 1992; Solc & Wine 1991; Valverde *et al.*, 1992; Worrell *et al.*, 1989) although other swelling-activated currents were not inactivated by voltage (Kelly *et al.*, 1994; Lewis *et al.*, 1993; Thiemaan *et al.*, 1992).

---

[2] A version of this chapter has been published. Ho MWY, Duszyk M & French AS (1994) Evidence that channels below 1 pS cause the volume-sensitive chloride conductance in T84 cells. *Biochim. Biophys. Acta.* 1191:151-156.

There is also an association between swelling-sensitivity and intracellular ATP levels. ATP was reported to be essential for development of the swelling-activated current in T-lymphocytes (Lewis *et al.*, 1993), insulin-secreting cells (Best *et al.*, 1996), and epithelial cells (Díaz *et al.*, 1994; Wu *et al.*, 1996). Intracellular ATP cross-reacted with cell swelling, calcium, and other agents in HT_{29} cells (Kunzelmann *et al.*, 1992), and was present in the pipet solution during several other studies (Kubo & Okada 1992; McCann *et al.*, 1989; Worrell *et al.*, 1989). However, ATP has also been reported to activate a low conductance chloride channel via a membrane-bound cGMP-dependent protein kinase, which was not related to cell swelling (Lin *et al.*, 1992).

Volume regulation is particularly important in cells that are constantly exposed to a wide range of osmotic challenges, such as the epithelial lining found in the colon. The T84 cell line is a transplantable human carcinoma cell line derived from a lung metastasis of a human colon carcinoma (Dharmsathaphorn *et al.*, 1984). The tumour tissue was serially transplanted into BALB/c nude mice to develop into a cell line. T84 cells retain many traits of epithelial cells (Dharmsathaphorn *et al.*, 1984). They grow as a monolayer with polarity. The plasma membrane on the apical (medium facing) side forms microvilli and also tight junction with the adjacent cells. Most important of all, T84 cells retain the vectorial electrolyte transport and the ability to respond to hormones or neurotransmitters (Dharmsathaphorn *et al.*, 1984). T84 cells show an increase in Cl^- secretion when exposed to osmotic challenge (Cliff & Frizzell, 1990; Worrell *et al.*, 1989). The T84 cell line thus provides a convenient model for studying volume-dependent Cl^- conductance.

Here, I re-examined the ionic basis and biophysical properties of the swelling-activated current in T84 cells, by imposing a transmembrane osmotic gradient, without intracellular ATP or Ca^{2+} to minimize cross-activation of other whole-cell currents.

3.1.2 Experimental data

On forming the whole-cell configuration, with normal Ringer solution in the bath and hypertonic pipet solution (Table 2.2.1 & 2.2.2), visible swelling was detectable within one minute and swelling-activated current developed slowly (Figs. 3.1.1A & 3.1.1B). Typical current-voltage relationships for the whole-cell

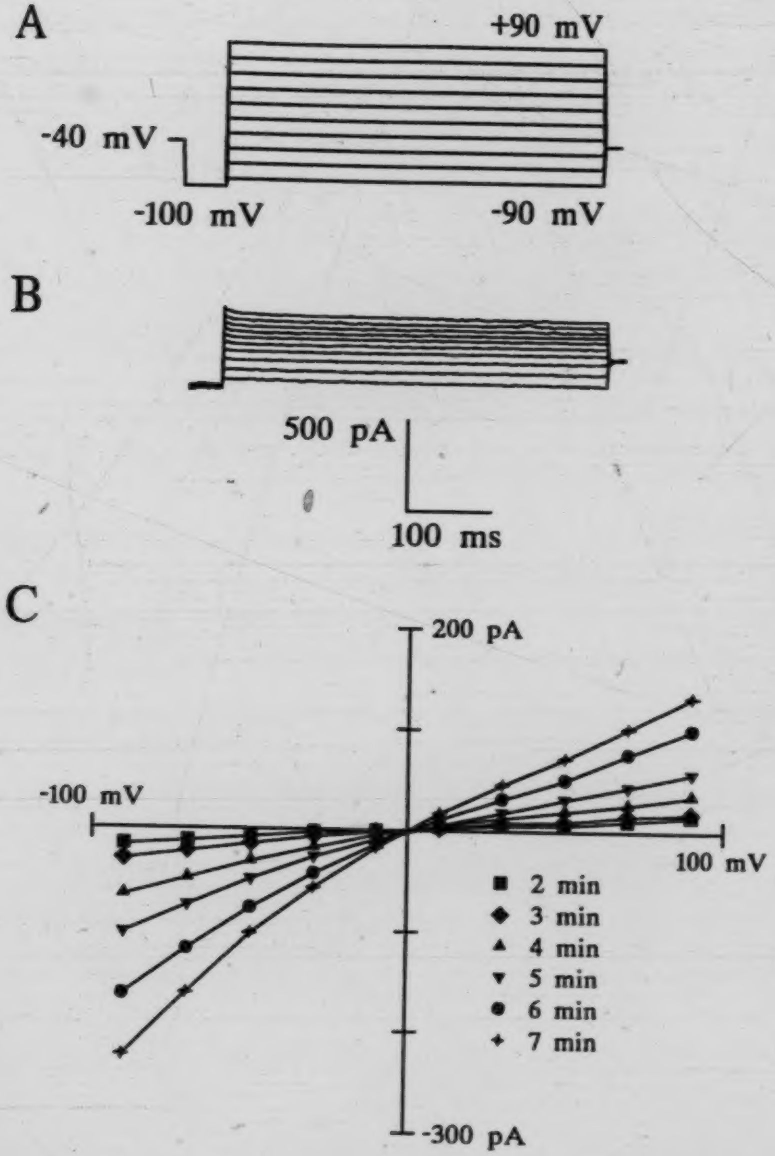

Figure 3.1.1. Development of the swelling-activated current after forming a whole-cell patch recording with normal bath Ringer and hypertonic pipet solution. (A) Voltage protocol used for all recordings, (B) original current traces recorded at the seventh minute in one cell, (C) steady state current-voltage relationships are shown for the total cell membrane current during the second to seventh minutes of the experiment from the same cell in (B). Note the inwardly rectifying nature of the current, and the reversal potential of zero at all times.

current during the first 7 minutes of a recording are shown in Fig. 3.1.1B. Currents like this appeared reliably without the presence of ATP in the pipet solution. The reversal potential for the currents was always near zero, close to the chloride equilibrium potential (~-0.2 mV) and far from either the sodium (+84 mV) or potassium (-84 mV) equilibrium potentials as estimated from Equation 2. No evidence was seen for inactivation of the current during steps from the initial potential of -100 mV to more depolarized potentials. Time-dependent activation or inactivation of the whole-cell current was also absent. Inward rectification was prominent throughout the development of the current.

During the development of the swelling-activated current, cells visibly swelled and eventually became unstable. Cell swelling and the swelling-activated current could be prevented by reversing the osmotic gradient, with the bath solution being hypertonic (Tables 2.2.1 & 2.2.2) (Fig. 3.1.2). Four minutes after starting the experiments, the mean current (±S.D.) at -90 mV for the swelling-activated whole-cell current was 108±45 pA (n=5) whereas the mean control current was 23±11 pA (n=5). The swelling-activated current was significantly larger than the control current ($p \leq 0.01$).

Detailed growth of the swelling-activated current was studied at a constant hyperpolarizing holding potential (-60 mV) due to the inwardly rectifying nature of its current-voltage relationship. A more hyperpolarizing voltage was not chosen to

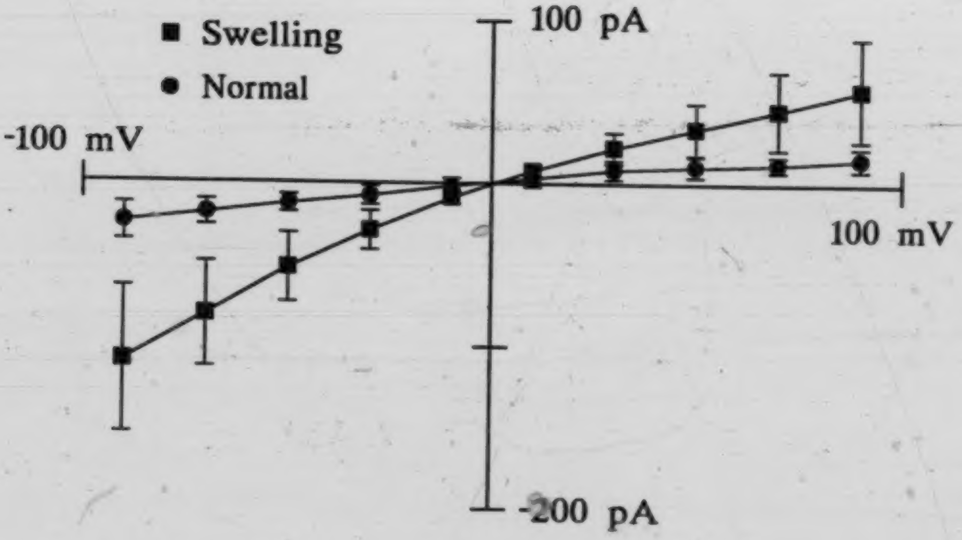

Figure 3.1.2. The appearance of the swelling-activated current could be prevented by reversing the osmotic gradient. Mean (±S.D.) current-voltage plots are shown from four whole-cell recordings in normal saline and hypertonic pipet solution (squares) and four cells in hypertonic saline and normal pipet solution (circles). All recordings were made four minutes after starting the experiments.

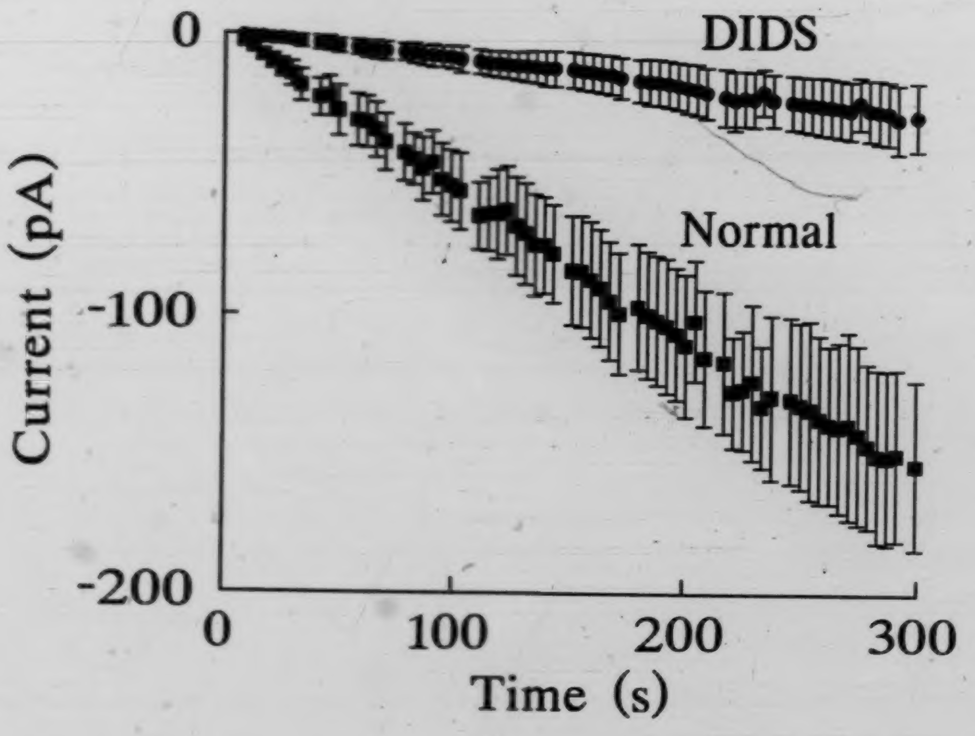

Figure 3.1.3. The swelling-activated current developed approximately linearly with time after forming the whole-cell patch and could be blocked by DIDS. Mean currents (± S.D.) at -60 mV holding potential are shown for ten cells in normal saline (squares) and seven cells in bath solution containing 20 μM DIDS (circles).

avoid possible damage to the seal and inaccuracy in recordings. The mean current (±S.D.) recorded from 10 different cells grew approximately linearly with time for five minutes after starting the whole-cell recordings (Fig. 3.1.3). Most of the swelling-activated current could be prevented by addition of 20 μM of the chloride channel blocker DIDS (Fig. 3.1.3). The mean current (±S.D.) for the swelling-activated current at the end of the recording was 153 ± 30 pA ($n=10$), which was significantly different than the current recorded with DIDS (28 ± 12 pA, $n=7$, $p\leq0.001$). Based on the reversal potential and its sensitivity to DIDS, this swelling-activated current in T84 cells was carried by chloride ions.

3.2 Characterization of the ion channels responsible for the swelling-activated Cl^- current [3]

3.2.1 Introduction

A wide variety of chloride single-channel conductances have been found in epithelia. For example, there are at least five different types of chloride channels in human nasal airway epithelia (Duszyk *et al.*, 1989) including channels of only 4-5 pS (Duszyk *et al.*, 1992; Wilk-Blaszczak *et al.*, 1992). The swelling-induced current in epithelia has been most commonly associated with an outwardly rectifying chloride channel with intermediate conductance between 40-75 pS (Banderali & Roy, 1992;

---

[3] A version of this chapter has been published. Ho MWY, Duszyk M & French AS (1994) Evidence that channels below 1 pS cause the volume-sensitive chloride conductance in T84 cells. *Biochim. Biophys. Acta.* 1191:151-156.

Kubo & Okada 1992; McCann *et al.*, 1989; Solc & Wine 1991; Worrell *et al.*, 1989), based on the outwardly rectifying nature of the current, observations of discrete current jumps in whole-cell records (Worrell *et al.*, 1989) and single channel records from swollen cells (Solc & Wine 1991). Swelling-activated Cl^- current has been also linked to the P-glycoprotein (Pgp), which produced strongly outwardly rectifying Cl^- currents when expressed in *Xenopus* oocytes (Gill *et al.*, 1992). However, the exact role of Pgp in volume regulation remains controversial.

Other swelling-activated Cl^- channels with large conductances (200-400 pS) have been reported in renal cortical collecting duct cells (Schwiebert *et al.*, 1994). Swelling-activated Cl^- channels with small conductance (< 5 pS) have also been described in large variety of cells including epithelia, using the noise analysis technique (Lewis *et al.*, 1993; Nilius *et al.*, 1994). In this section I characterize the Cl^- channels underlying the swelling-activated current in T84 cells, using the noise analysis technique.

3.2.2 Experimental data

Development of the swelling-activated current was accompanied by an increase in the noise variance of the current record, as would be expected if the current were due to an increase in the open probability of a group of ion channels from an initially low value. Figure 3.2.1A shows portions of the actual current traces at the beginning and the end of one recording. The noise level in the first minute was noticeably lower than in the fifth minute, indicating an increase in channel open probability during swelling. In all cases this noise variance could be well-fitted by the double Lorentzian function of Equation (3). The contribution of the low frequency Lorentzian component remained approximately constant, while the growth of the high frequency component accounted for most of the increase in total variance. Figure 3.2.1B shows the current noise spectra obtained during the first and fifth minutes of a typical experiment. During the first minute, the two components contributed variances of 0.46 pA^2 and 0.36 pA^2 with corner frequencies of 1.7 Hz and 62.7 Hz respectively. By the fifth minute the first component was unchanged but the second component had increased to 2.06 pA^2, with a corner frequency of 53.2 Hz.

Similar Lorentzian fits were obtained from the first and fifth minutes of current development in 10 different cells. The mean (± S.D.) of the total current noise variance after 5 minutes was 1.93 ± 0.51 pA^2 with the relative contribution of the second component being $70.4 \pm 0.06\%$. The mean corner frequencies (± S.D.) of the two components were: 1.72 ± 0.52 Hz and 59.27 ± 7.5 Hz respectively. Therefore, the two Lorentzian components could be reliably identified and always made about the same relative contributions to the total noise variance. For the 10 experiments, the mean corner frequency of the low frequency component did not change significantly during 5 minutes of swelling (1.75 to 1.72 Hz), but the corner frequency of the high

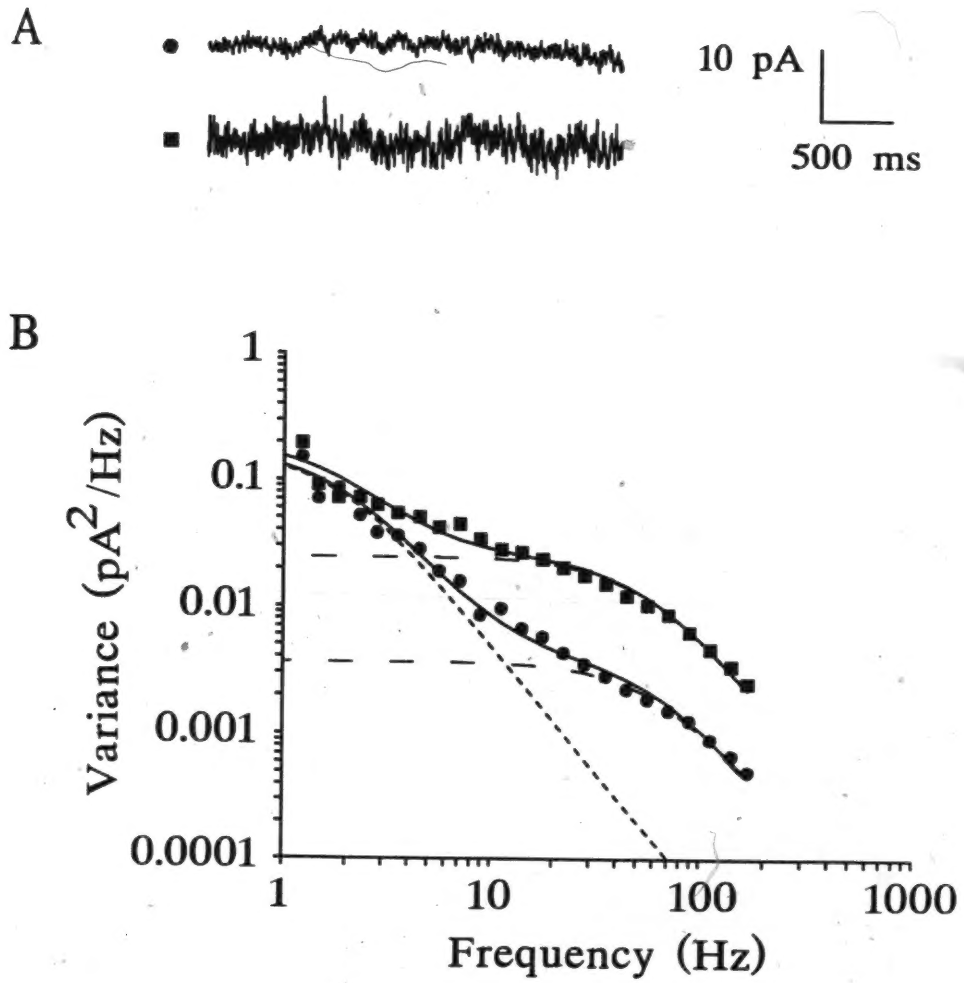

Figure 3.2.1. Membrane noise variance increased during development of the swelling-activated current, at a holding potential of -60 mV. (A) Portions of the actual current records are shown for the first minute (circles) and fifth minute (squares) of a typical current recording, (B) Noise spectra are shown for these periods from the same cell. Both spectra were fitted by double Lorentzian functions (Equation 3). For the first minute, the fitted parameters were: $S_1=0.17$ pA^2/Hz, $\theta_1=1.7$ Hz, $S_2=0.004$ pA^2/Hz, $\theta_2=62.7$ Hz. For the fifth minute, the fitted parameters were: $S_1=0.18$ pA^2/Hz, $\theta_1=1.7$ Hz, $S_2=0.025$ pA^2/Hz, $\theta_2=53.2$ Hz. The two low frequency components completely overlapped (short dashed lines). The high frequency components are shown as long dashed lines and the summed components as solid lines.

frequency component decreased by about 25% (76.06 to 59.27 Hz).

Equation (4) predicts that the noise variance, σ^2_s produced by ion channels with changing open probability will be a second-order polynomial function of total membrane current, I. When the channels are totally closed (P_o=0) or open (P_o=1) the variance will be zero, but it will reach a maximum of I^2/N when P_o=0.5. Noise variance did increase during development of the swelling-activated current (Fig. 3.2.1B) and its dependence on the current could be approximated by Equation (4). Figure 3.2.2 shows this relationship for the first 5 minutes of one recording, with the fitted line corresponding to 32,190 channels of 0.25 pS conductance. Similar measurements were made on 10 different cells, with mean (±S.D.) parameters of: $\sigma_0{}^2$=0.43±0.23 pA^2, γ=0.20±0.04 pS, and N=89,000±98,000 channels. During the experiments with 20 μM DIDS in the bath, the total noise variance did not exceed 0.5 pA^2, close to the background variance.

The open probabilities of the channels were calculated as functions of time from Equation (5) during each experiment. Mean values of P_o versus time increased from near zero at the start of each experiment to a maximum of 0.25 after 5 minutes (Fig. 3.2.3). However, these data were quite variable, with some individual cells showing open probabilities of up to 0.6 after five minutes.

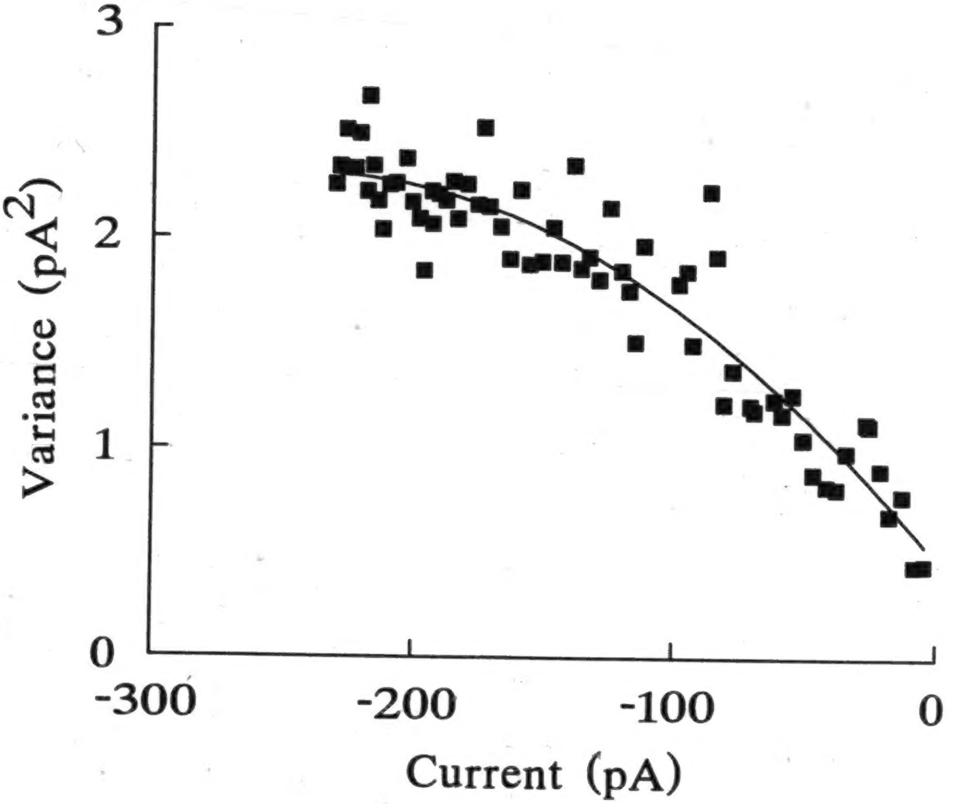

Figure 3.2.2. Membrane noise variance as a function of total membrane current during the development of the swelling-activated current. Squares show the original variance data. The fitted line is from Equation (4) with parameters: σ_0^2=0.48 pA^2, γ=0.25 pS, and N=32,190 channels for this cell.

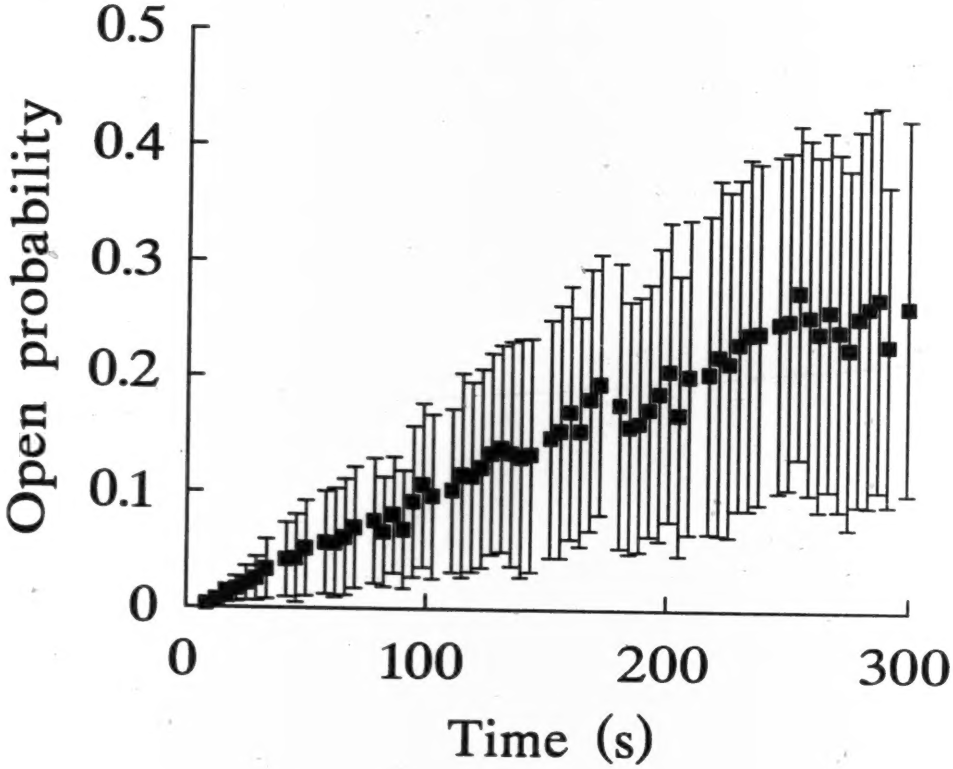

Figure 3.2.3. Calculated open probability as a function of time after forming the whole-cell patch. Mean (± S.D.) values are shown for the same ten cells as in Fig. 3.1.3.

3.3 Identification of surface purinergic receptors in CFPAC-1 cells

3.3.1 Introduction

Extracellular ATP exerts its effects on a variety of systems via surface P_2 purinoceptors (Dubyak & El-Moatassim 1993). Several P_2 purinoceptors have been identified either as directly ATP-gated channel (P_{2X}) or pores (P_{2Z}), or as G-protein coupled receptors (P_{2T}, P_{2Y} and P_{2U}). Classification of these purinoceptors is based on the potency of different analog agonists (Dubyak & El-Moatassim 1993). Expression of some of these receptors is quite tissue specific, with an abundant expression of P_{2X} in excitable and secretory cells, P_{2Z} in macrophages, mast cells and lymphocytes, and P_{2T} in platelets (Dubyak & El-Moatassim 1993). However, P_{2Y} and P_{2U} have been reported in many cell types including epithelia (Klär et al., 1993; Nilius *et al.*, 1995b; Stutts *et al.*, 1994). The overall effect of ATP, despite its diverse receptor types, is to increase cytosolic Ca^{2+} and therefore regulate all Ca^{2+}-dependent cellular processes (Dubyak & El-Moatassim 1993).

Epithelial cells from CF patients lack the cAMP-regulated Cl^- channel but retain a separate functional Ca^{2+}-dependent regulatory pathway for Cl^- secretion (Boucher *et al.*, 1989). Recent studies have suggested that ATP or UTP could be used as a therapeutic treatment for CF, due to their ability to activate PLC and subsequently increase intracellular Ca^{2+} (Knowles *et al.*, 1995). In CF airway epithelial cells, adenosine (Chao *et al.*, 1994; Lazarowski *et al.*, 1992), ATP and UTP (Clarke & Boucher 1992; Mason *et al.*, 1991; Stutts *et al.*, 1994) all show regulatory effects on Cl^- secretion. Furthermore, P_{2X} receptor agonists increase Cl^- conductance in CFTR transfected cells (Cantiello *et al.*, 1994). In spite of their common abilities to increase Cl^- secretion in CF epithelium, each of these agonists has a different cellular mechanism. It is therefore important to identify the P_2 receptors and the cellular pathways involved, in order to improve the therapeutic treatment of CF.

CFPAC-1 cells, which were developed from a CF patient with the ΔF508 defect, have been used to study ion transport in CF epithelium (Schoumacher *et al.*, 1990). It has been shown that CFPAC-1 cells express different surface receptors for various Ca^{2+}-mobilizing agonists including ATP (Klär *et al.*, 1993). Thus, CFPAC-1 cells are a good model for studying the P_2 receptors in CF epithelia. In this section I identify the P_2 receptor expressed in CFPAC-1 cells by measuring intracellular Ca^{2+} ($[Ca^{2+}]_i$) changes, indicated as changes in fluorescence ratio from a calcium-sensitive dye, with different P_2 agonists.

3.3.2 Experimental data

A biphasic change in $[Ca^{2+}]_i$ was induced by 10 μM extracellular ATP in CFPAC-1 cells (Fig. 3.3.1). Intracellular Ca^{2+} increased to its peak value within 10-15 s after application of ATP. $[Ca^{2+}]_i$ was then reduced to a lower but still

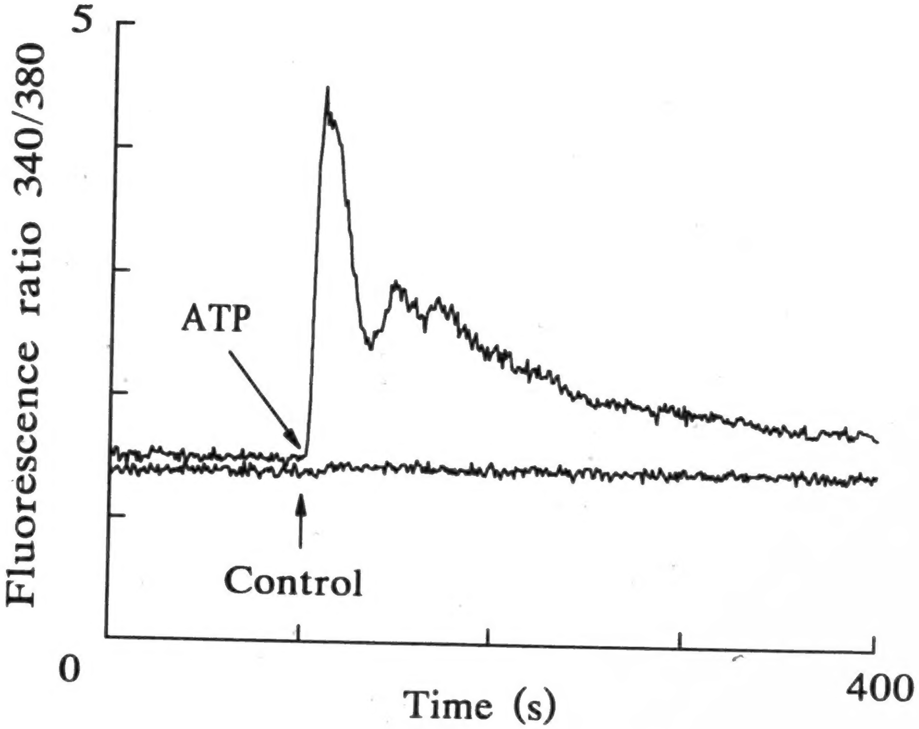

Figure 3.3.1. ATP induced a biphasic increase in $[Ca^{2+}]_i$. Intracellular $[Ca^{2+}]$ was measured from the Fura-2 pre-loaded CFPAC-1 cells, as a ratio of fluorescence intensity measured over 400 nm with excitation wavelengths of 340 nm and 380 nm. Extracellular 10 μM ATP induced an initial transient increase in $[Ca^{2+}]_i$ within ten to fifteen seconds in the normal bath solution. The level of $[Ca^{2+}]_i$ then declined to a lower, but still elevated level and slowly decayed towards the baseline level. Application of the same amount of water as a control failed to evoke any changes in the fluorescence ratio. Traces represent typical recordings from three to six experiments.

elevated level for 50-100 s before slowly returning towards the baseline level. In contrast to the initial transient response, the second slow or plateau Ca^{2+} response was quite variable. When an equal amount of water was applied as a control experiment (Fig. 3.3.1), no change in $[Ca^{2+}]_i$ was observed, indicating that the change in $[Ca^{2+}]_i$ associated with ATP was not due to a mechanical artifact. ATP mobilized Ca^{2+} in a dose-dependent manner in CFPAC-1 cells, at levels as low as 200 nM (Fig. 3.3.2). Beside the difference in the peak response of $[Ca^{2+}]_i$, low levels of ATP increased the time constants for the rise and fall of the transient peak $[Ca^{2+}]_i$.

When extracellular Ca^{2+} was removed from the bath solution, the peak $[Ca^{2+}]_i$ response upon ATP challenge showed no significant difference to that recorded in the normal bath solution (data not shown). The mean increases in fluorescence ratio (±S.E.) were 2.92±0.06 (n=6) and 3.02±0.11 (n=5) for recordings with 2 mM and 0 mM extracellular Ca^{2+}, respectively. When intracellular Ca^{2+} stores were depleted by 70 nM TG, no change of $[Ca^{2+}]_i$ could be induced by ATP (Fig. 3.3.3). These experiments indicate that activation of surface P_2 receptors by ATP caused the mobilization of intracellular Ca^{2+}. Finally, extracellular UTP was equipotent with ATP in increasing $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} (Fig. 3.3.4). Both the transient response and the slow decay in ATP- and UTP-stimulated cells showed similar time courses. The mean absolute increase in fluorescence ratio (±S.E.) was 3.03±0.14 (*n*=4) with UTP, not significantly different from the ATP-induced ratio change (3.02±0.11, *n*=5).

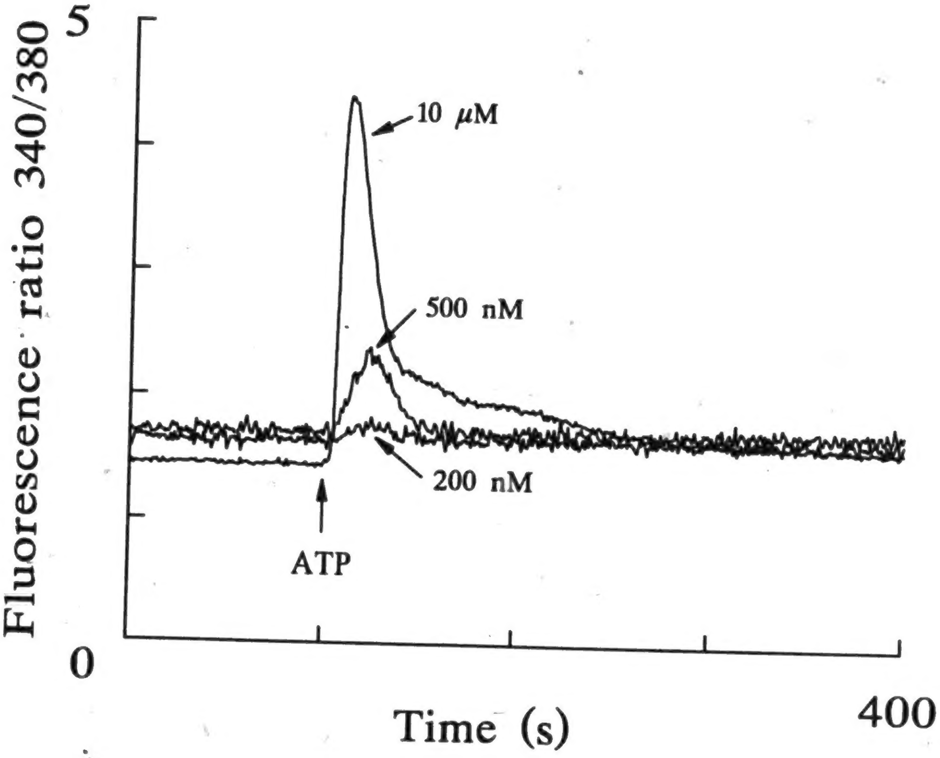

Figure 3.3.2. ATP caused a dose-dependent increase in $[Ca^{2+}]_i$. CFPAC-1 cells showed sensitivity to extracellular ATP at concentrations as low as 200 nM. Increased ATP concentrations induced faster changes in the initial response. Traces represent typical recordings from four to six experiments.

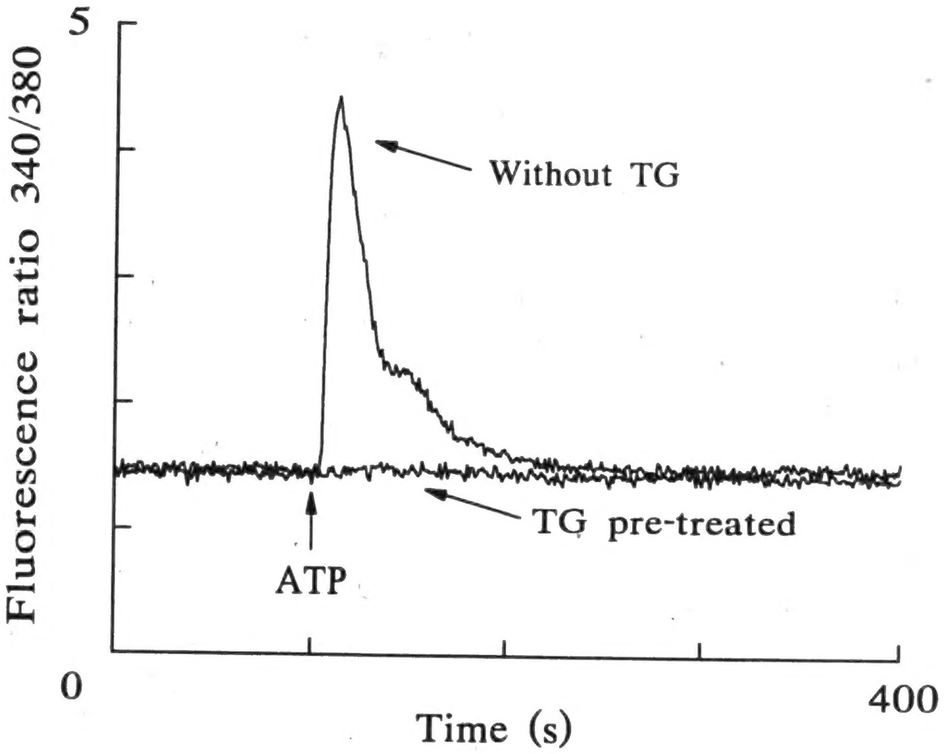

Figure 3.3.3. Thapsigargin prevented the ATP-mediated Ca^{2+} response in CFPAC-1 cells. CFPAC-1 cells were pre-treated with 70 nM thapsigargin in culture medium and subsequently bathed in zero-Ca^{2+} solution for five minutes before application of ATP. ATP (10 μM) was no longer effective in increasing $[Ca^{2+}]_i$ after TG treatment. Traces represent typical recordings from four to five experiments.

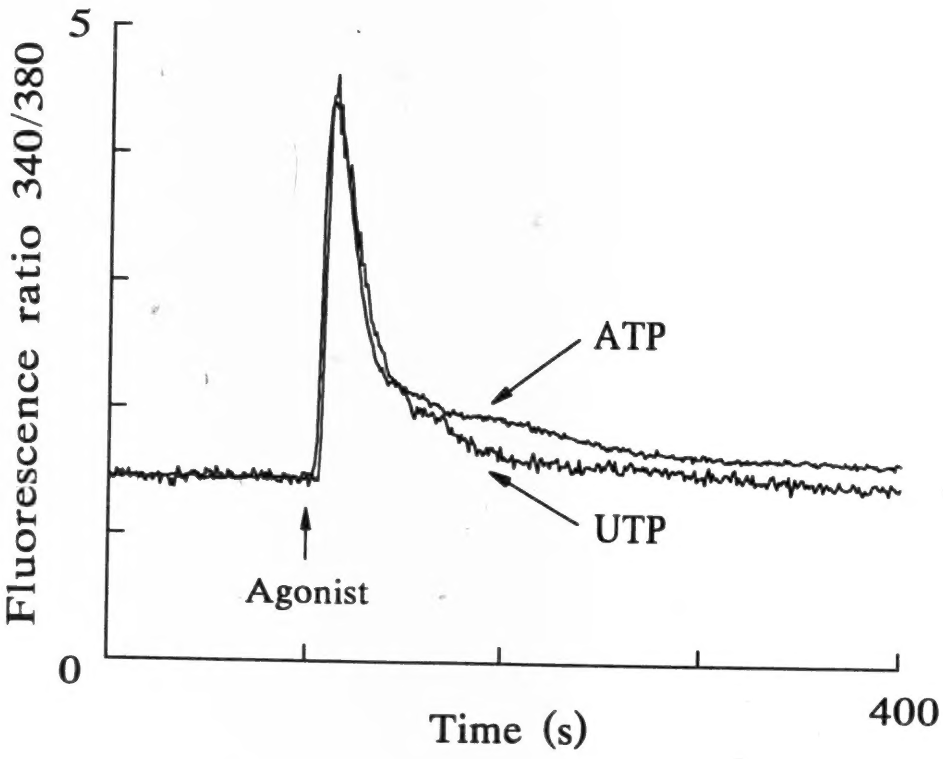

Figure 3.3.4. CFPAC-1 cells were sensitive to extracellular UTP as well as ATP. 10 μM of extracellular UTP induced a similar transient Ca^{2+} response in CFPAC-1 cells to the same concentration of ATP. Both recordings were done in the absence of extracellular Ca^{2+}. Traces represent typical recordings from four to five experiments.

3.4 Ionic basis of the Ca^{2+}-dependent current in CFPAC-1 cells [4]

3.4.1 Introduction

Calcium is an important intracellular signalling molecule for many cellular functions, such as neurotransmitter release, muscle contraction and hormone secretion. In epithelial cells, Ca^{2+} plays a major role in regulating ion transport, especially in CF where the cAMP regulation is defective (Boucher *et al.*, 1989). Enhancement of this Ca^{2+}-dependent alternative regulatory pathway for ion transport by extracellular agonists has been proposed as a potential therapeutic treatment for CF (Knowles *et al.*, 1995). One such agonist is ATP and its potential in CF treatment has been thoroughly reviewed (Jiang *et al.*, 1993; Knowles *et al.*, 1995).

CFPAC-1 cells lack a cAMP-activated chloride current, and only express the remaining types of Cl^- currents, the swelling-induced current, and the agonist-mediated, Ca^{2+}-dependent current (Schoumacher *et al.*, 1990; Warth & Greger 1993). Thus, CFPAC-1 cells provide a good model for studying Ca^{2+}-mediated ion transport in CF. In the previous section, it was shown that there is a purinergic receptor expressed in CFPAC-1 cells, most likely the P_{2U} subtype. Here I examine the effect of increased $[Ca^{2+}]_i$ induced by P_2 receptor activation on Cl^- ion movement in CFPAC-1 cells using the whole-cell patch clamp technique.

---

[4] **A version of this chapter has been accepted for publication. Ho MWY, Shears SB, Bruzik K, Duszyk M & French AS: Ins(3,4,5,6)P_4 specifically inhibits a receptor-mediated, Ca^{2+}-dependent Cl^- current in CFPAC-1 cells *Am. J. Physiol. (Cell Physiol.)***

3.4.2 Experimental data

The voltage protocol and control whole-cell currents in the absence of extracellular ATP are shown in Figs. 3.4.1A and 3.4.1B. When 2 μM ATP was added to the bath solution, an outwardly rectifying whole-cell current developed (Fig. 3.4.1C). This concentration of ATP is within the dose range that elicited maximal responses in an earlier study of CFPAC-1 cells (Warth & Greger 1993). Fig. 3.4.2 shows typical current-voltage relationships over a four minute recording period after the addition of ATP. The ATP-activated whole-cell current showed strong outward rectification in symmetric chloride solutions and slow activation at a constant voltage, with the activation being stronger at more depolarizing voltages. The reversal potential of the whole-cell current was near zero millivolts, which was close to the equilibrium potential for Cl^- (~-0.2 mV) as estimated from Equation (2).

Unlike the transient nature of the ATP-activated current in other cell types (Jiang *et al.*, 1993), the ATP-activated whole-cell current developed progressively over time in CFPAC-1 cells (Figs. 3.4.2 & 3.4.4). The mean current (±S.E.) at +90 mV slowly increased up to 5-fold during these experiments, with an average maximum current of 247±26 pA ($n=7$). ATP-activated current became significantly ($p \leq 0.001$,

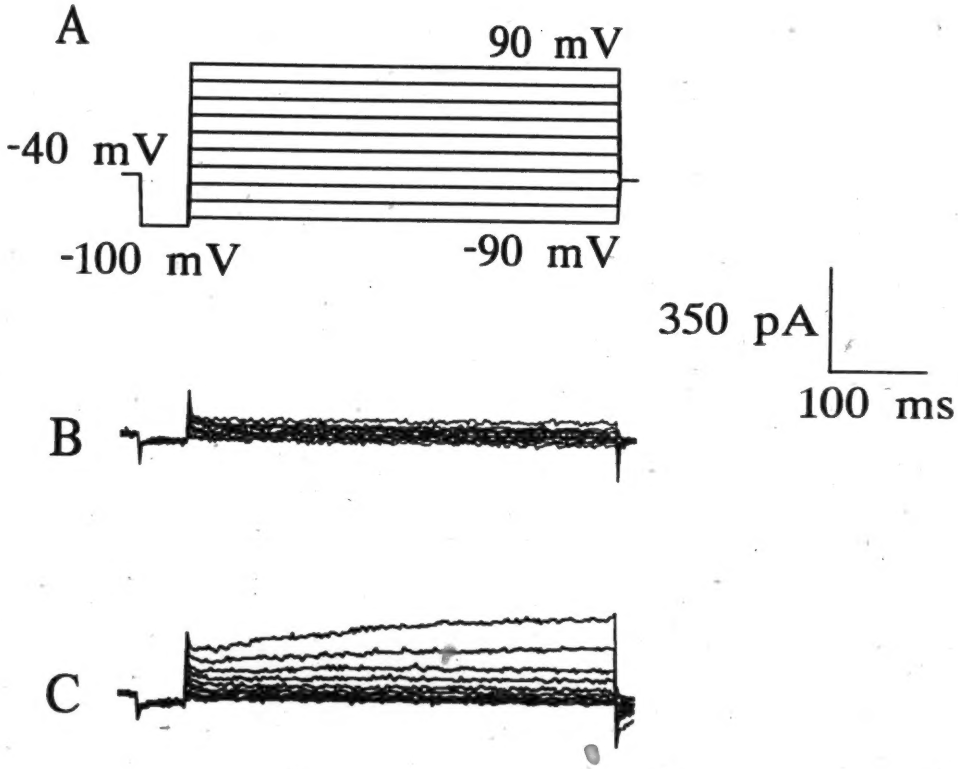

Figure 3.4.1. Extracellular ATP elevated the whole-cell current in CFPAC-1 cells. (A) the voltage protocol used for all recordings, (B) whole-cell current traces recorded without extracellular ATP, (C) typical current induced by 2 μM extracellular ATP. Current traces from both (B) and (C) were recorded four minutes after forming the whole-cell and the ATP application, respectively.

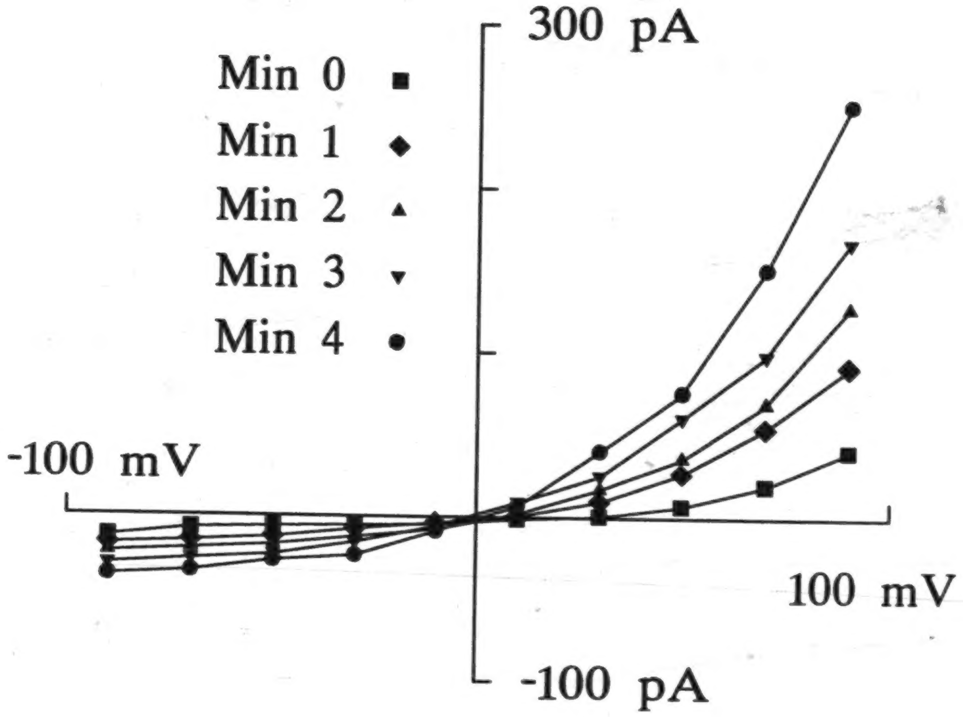

Figure 3.4.2. Current-voltage relationship of the ATP-activated whole-cell current. Current-voltage relationships obtained from the same cell described in Fig. 3.4.1C, during first four minutes after the addition of 2 μM ATP to the bath solution. The current showed strong outward rectification in symmetric chloride solutions (see Tables 2.2.1 & 2.2.2).

n=6-7) larger than the mean baseline current (n=6) two minutes after the application of ATP.

Development of the ATP-activated current was strongly reduced by the presence of the chloride channel blocker DIDS (50 μM) in the bath solution (Fig. 3.4.3A). Following DIDS application, the current declined and its reversal potential shifted towards the equilibrium potential for potassium (-85 mV, Eq. 2) (Fig. 3.4.3B). Three minutes after applying ATP, the mean current (±S.E.) recorded with DIDS in the bath at +90 mV was 74±14 pA (n=5), with a reversal potential of -22 mV. This residual current was significantly smaller than the current recorded with only 2 μM ATP (242±26, n=7, $p \leq 0.01$) and represented about 14% of the total ATP-stimulated whole-cell current. It was probably carried by K^+.

There is evidence that in certain cell types, extracellular ATP can stimulate an outwardly rectifying Cl^- current independently of changes in intracellular Ca^{2+} levels (Schwiebert *et al.*, 1995; Stutts *et al.*, 1994). I therefore measured the ATP-activated Cl^- current when the extracellular and intracellular solutions contained essentially no free Ca^{2+}. Figure 3.4.4A shows a typical whole-cell current recorded under zero-Ca^{2+} conditions. Under these conditions, ATP was no longer effective in evoking a whole-cell Cl^- current even three minutes after application. The effects of ATP on whole-cell current under zero-Ca^{2+} conditions were not significantly different from the control current throughout the recording period, whereas under normal recording

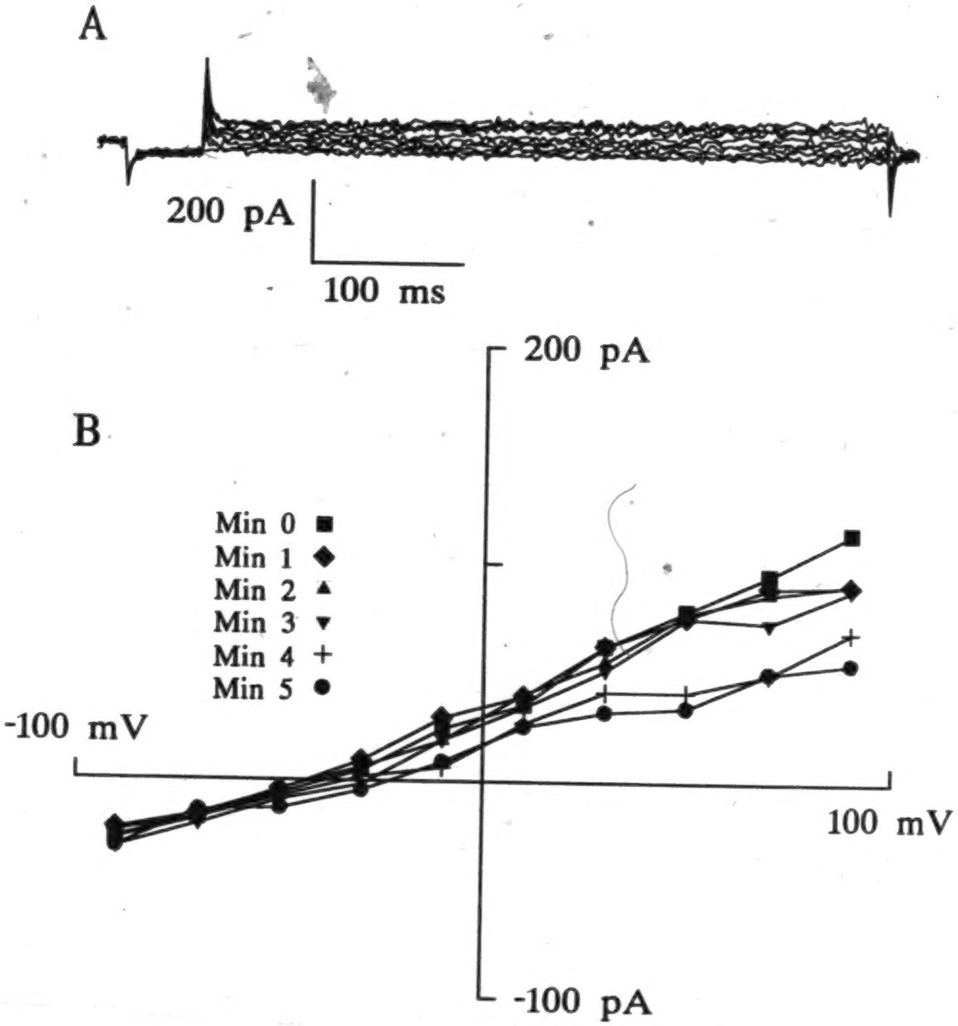

Figure 3.4.3. The chloride channel blocker, DIDS, reduced the ATP-activated whole-cell current. (A) Current recorded with 50 μM extracellular DIDS, four minutes after application of 2 μM extracellular ATP to the bath solution and (B) current-voltage relationships obtained from the same cell as (A). In this recording, the current measured at +90 mV after four minutes stimulation with extracellular ATP was 69 pA. The current reversed at -34 mV. Three minutes after the application of ATP, mean current at +90 mV (±S.E.) was 74±14 pA (n=5). The reversal potential was then -22 mV.

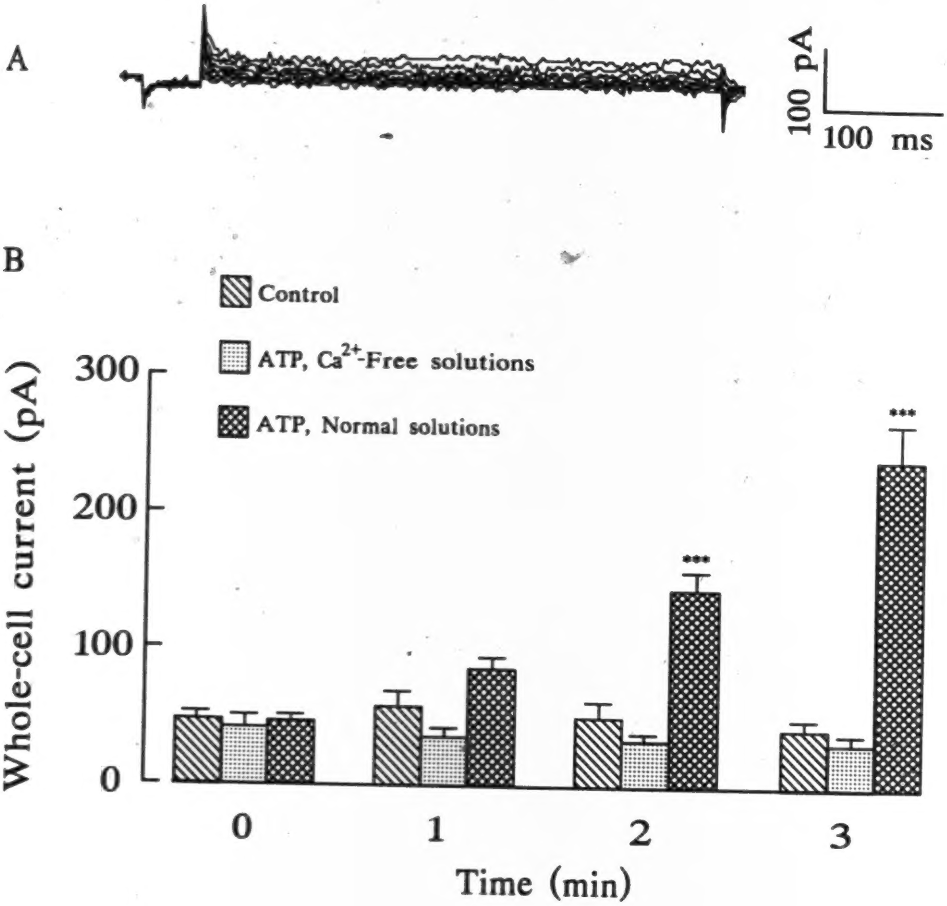

Figure 3.4.4. ATP evoked a Ca^{2+}-dependent whole-cell Cl$^-$ current. (A) Typical whole-cell current induced by 2 μM extracellular ATP under zero-Ca^{2+} conditions, four minutes after ATP application, and (B) development of mean whole-cell current (±S.E.) at +90 mV in control experiments with normal solutions, 2 μM ATP recorded with zero-calcium solutions and 2 μM ATP recorded with normal solutions. After removing all intra- and extra-cellular Ca^{2+}, ATP was no longer able to induce the outwardly rectifying Cl$^-$ current. Number of experiments was between five and seven.

conditions ATP effects became evident ($p \leq 0.001$, $n=6$-7) by the second minute (Fig. 3.4.4B).

The calcium-dependence of the ATP-activated Cl^- current could also be demonstrated by elevating cytosolic Ca^{2+} with the ATPase inhibitor thapsigargin (TG). An outwardly rectifying whole-cell Cl^- current was activated in CFPAC-1 cells by 1 μM extracellular TG (Fig. 3.4.5A). This TG-activated current had similar electrophysiological properties to the ATP-activated current, including slow activation at constant voltage (Fig. 3.4.5A) and a reversal potential near 0 mV (Fig. 3.4.5B). TG increased the mean whole-cell current at +90 mV to 328±53 pA (±S.E., $n=5$) three minutes after application, and this current was significantly greater ($p \leq 0.05$) than the ATP-activated Cl^- current (242±26, $n=7$).

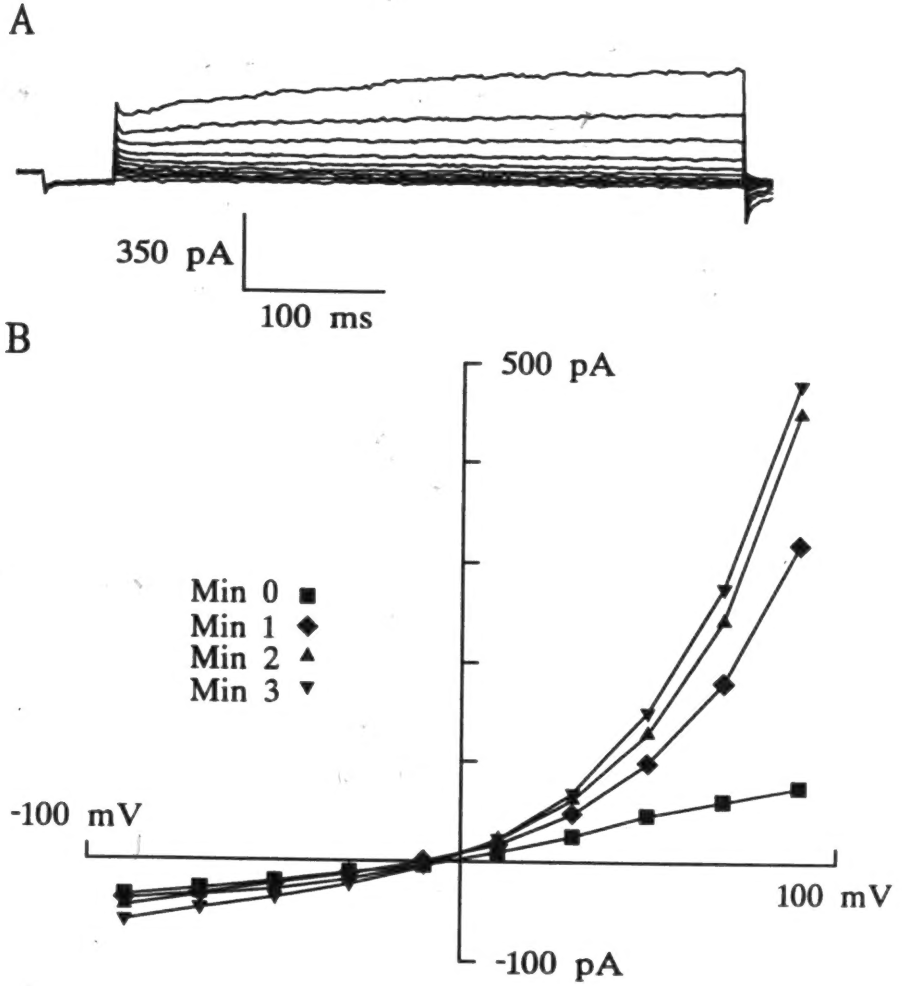

Figure 3.4.5. Thapsigargin induced a whole-cell current that was similar to the ATP-activated whole-cell Cl^- current. (A) Typical whole-cell current induced by 1 μM TG, three minutes after application, and (B) current-voltage relationships obtained from the same cell as (A). The TG-induced whole-cell current had similar biophysical properties like outward rectification and slow activation at depolarizing voltages to the current induced by ATP.

3.5 Regulation of the Ca^{2+}-dependent Cl^- current by inositol phosphates [5]

3.5.1 Introduction

Activation of PLC via surface receptors such as P_{2Y} and P_{2U} purinoceptors causes a rapid hydrolysis of membrane phosphatyinositol phosphates and generates Ins(1,4,5)P_3 and diacylglycerol (DG) (Berridge & Irvine 1989). Ins(1,4,5)P_3 mobilizes Ca^{2+} from intracellular stores, whereas DG, in the presence of Ca^{2+}, activates PKC. The combined actions of these two intracellular signal molecules are responsible for regulating all known Ca^{2+}-dependent cellular processes. However, evidence has emerged to show that the inositol phosphate cycle is complex and that some inositol phosphates, other than Ins(1,4,5)P_3, might also have cellular functions. The first such inositol phosphate to be investigated was Ins(1,3,4,5)P_4, which was shown to mobilize Ca^{2+} from Ins(1,4,5)P_3-sensitive pools (DeLisle *et al.*, 1994; Ivorra *et al.*, 1991) and/or to regulate Ca^{2+} influx (Irvine 1992; Lückhoff & Clapham 1992) upon receptor activation. Other InsP_4s such as Ins(1,3,4,6)P_4 and Ins(1,4,5,6)P_4 have been shown to mobilize Ca^{2+} from Ins(1,4,5)P_3-sensitive pools (DeLisle *et al.*, 1994; Ivorra *et al.*, 1991; Lu *et al.*, 1996), and therefore might also regulate the overall Ca^{2+} signal.

---

[5] A version of this chapter has been accepted for publication. Ho MWY, Shears SB, Bruzik K, Duszyk M & French AS: Ins(3,4,5,6)P_4 specifically inhibits a receptor-mediated, Ca^{2+}-dependent Cl^- current in CFPAC-1 cells *Am. J. Physiol. (Cell Physiol.)*

Activation of PLC and the subsequent increase in intracellular Ca^{2+} promotes Cl^- secretion in epithelia (Clarke & Boucher 1992; Stutts *et al.*, 1994). However, the use of these Ca^{2+}-dependent regulatory pathways to compensate for defective Cl^- secretion has been limited. Activation of PLC in intestinal epithelia promotes an inhibitory feedback process that prevents Ca^{2+}-dependent Cl^- secretion from being sustained, even in the continual presence of the agonist, limiting the efficacy of this therapeutic approach (Jiang *et al.*, 1993). These authors also showed that salt and fluid secretion across nasal epithelial layers can only be transiently elevated by UTP (Jiang *et al.*, 1993). Greater understanding of the molecular mechanisms of this desensitization could potentially lead to its circumvention and an improved CF therapy.

The first evidence suggesting that InsP_4 might be involved in the inhibition of Ca^{2+}-dependent Cl^- secretion came from a biochemical study (Kachintorn *et al.*, 1993). Subsequently, attention began to focus on a particular isomer of InsP_4, Ins(3,4,5,6)P_4. Intracellular concentrations of this polyphosphate increase substantially during prolonged activation of PLC, due to a receptor-mediated increase in the rate of dephosphorylation of Ins(1,3,4,5,6)P_5 (Fig. 1.6) (Kachintorn *et al.*, 1993; Menniti *et al.*, 1990; Oliver *et al.*, 1992), and this was found to coincide with the inhibition of chloride secretion (Kachintorn *et al.*, 1993; Vajanaphanich *et al.*, 1994). Then Ins(3,4,5,6)P_4 was found to inhibit Ca^{2+} stimulated Cl^- secretion in the T84 colonic epithelial cell line, by the use of a cell-permeant analog of this polyphosphate

(Vajanaphanich *et al.*, 1994) and by electrophysiological experiments (Xie *et al.*, 1996).

To date, Ins(3,4,5,6)P_4 has only been shown to affect Cl^- transport in T84 cells. Therefore, it is particularly important to see if the phenomenon occurs in a different cell type. In this respect, we anticipated that the CFPAC-1 cell line would be a particularly useful model. This cell line was originally derived from pancreatic ductal carcinoma cells of a CF patient suffering from the ΔF508 defect (Schoumacher *et al.*, 1990). The Cl^- current in these cells is not affected by cAMP but remains responsive to stimulation by extracellular agonists that mobilize intracellular Ca^{2+} (Warth & Greger 1993). Furthermore, responses to Ins(3,4,5,6)P_4 in CFPAC-1 cells should be a good reflection of such regulation in CF epithelium. Thus, this cell type offers the opportunity to study Cl^- transport completely independently of the CFTR pathway. In this section I will describe the regulatory actions of different inositol tetrakisphosphates, in particular the inhibitory effect of Ins(3,4,5,6)P_4 on Cl^- conductance in epithelial cells, using the whole-cell patch clamp.

3.5.2 Experimental data

All the agonists that activate PLC, including ATP, elevate cytosolic levels of Ins(3,4,5,6)P_4 through receptor-dependent regulation of the Ins(1,3,4,5,6)P_5 1-phosphatase/Ins(3,4,5,6)P_4 1-kinase substrate cycle (Menniti *et al.*, 1990; Oliver *et* *al.*, 1992). Basal levels of Ins(3,4,5,6)P_4 are about 1 μM, and subsequently rise to about 10 μM after prolonged receptor activation (Vajanaphanich *et al.*, 1994). Therefore, I first measured ATP-activated Cl$^-$ currents in CFPAC-1 cells after application of 10 μM Ins(3,4,5,6)P_4 by diffusion through the recording pipet. This concentration of Ins(3,4,5,6)P_4 completely prevented ATP from stimulating the Cl$^-$ current (Figs. 3.5.1A & 3.5.1B). The inhibitory effect of Ins(3,4,5,6)P_4 on ATP-activated Cl$^-$ currents became significant ($p \leq 0.001$) two minutes after the application of ATP (Fig. 3.5.2). When recording conditions permitted, inhibition of ATP-activated Cl$^-$ current by Ins(3,4,5,6)P_4 lasted as long as 8 minutes (data not shown).

The inhibitory effect of Ins(3,4,5,6)P_4 was dose-dependent, with a majority of the ATP-activated Cl$^-$ current being blocked by a concentration as low as 4 μM (Fig. 3.5.3). A further decrease in Ins(3,4,5,6)P_4 concentration led to a sharp reduction in the inhibition and 2 μM Ins(3,4,5,6)P_4 had no significant effect on Cl$^-$ current. The steepness of this dose/response curve is reflected in the relatively high co-operativity value (7.14) that was obtained by fitting Equation (1) to the data. The estimated apparent epuilibrium constant for the inhibitory action of Ins(3,4,5,6)P_4 was 2.86 μM.

Previous work has demonstrated that, in T84 cells, Ins(3,4,5,6)P_4 inhibits the Ca^{2+}-dependent Cl$^-$ conductance independently of the process of receptor-mediated

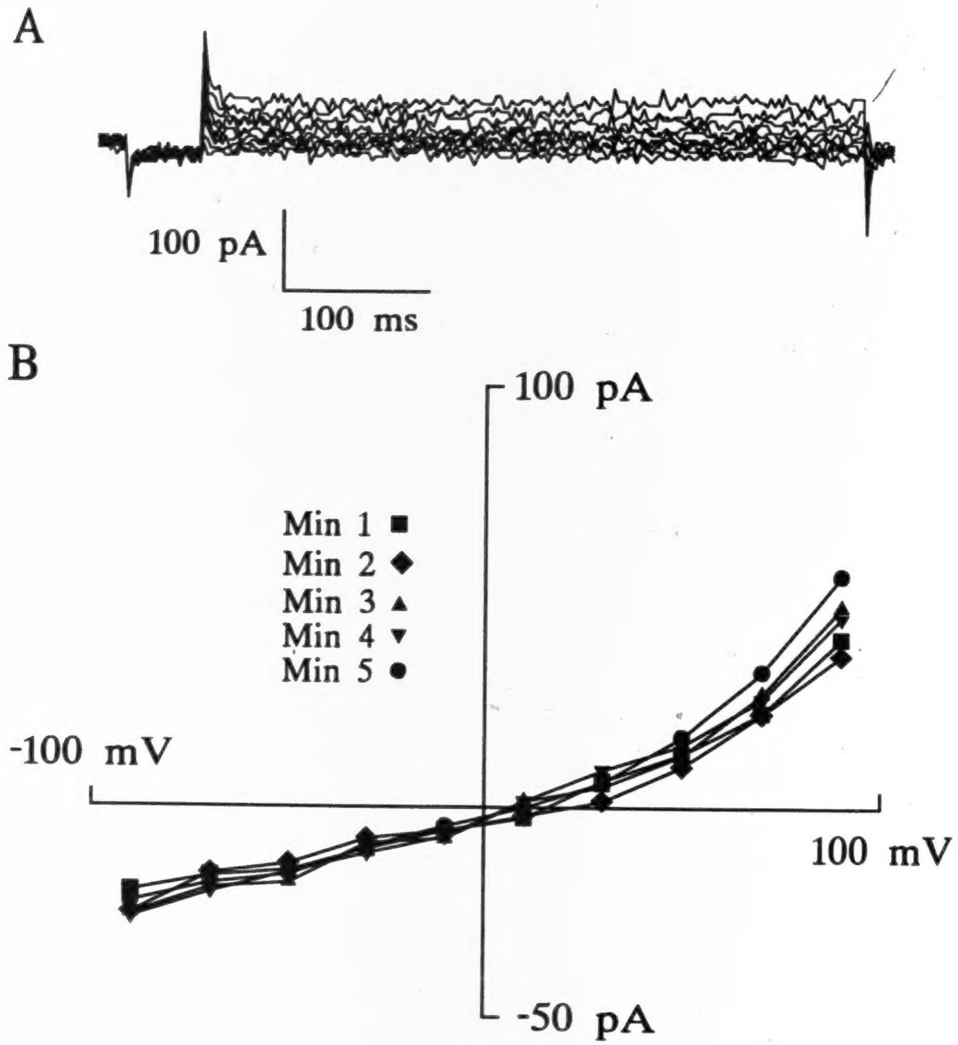

Figure 3.5.1. Intracellular Ins(3,4,5,6)P_4 inhibited the ATP-activated Ca^{2+}-dependent Cl^- current. (A) Typical whole-cell currents recorded with 10 μM Ins(3,4,5,6)P_4 in the pipet solution, four minutes after addition of 2 μM extracellular ATP to the bath solution, (B) current-voltage relationships obtained from the same cell.

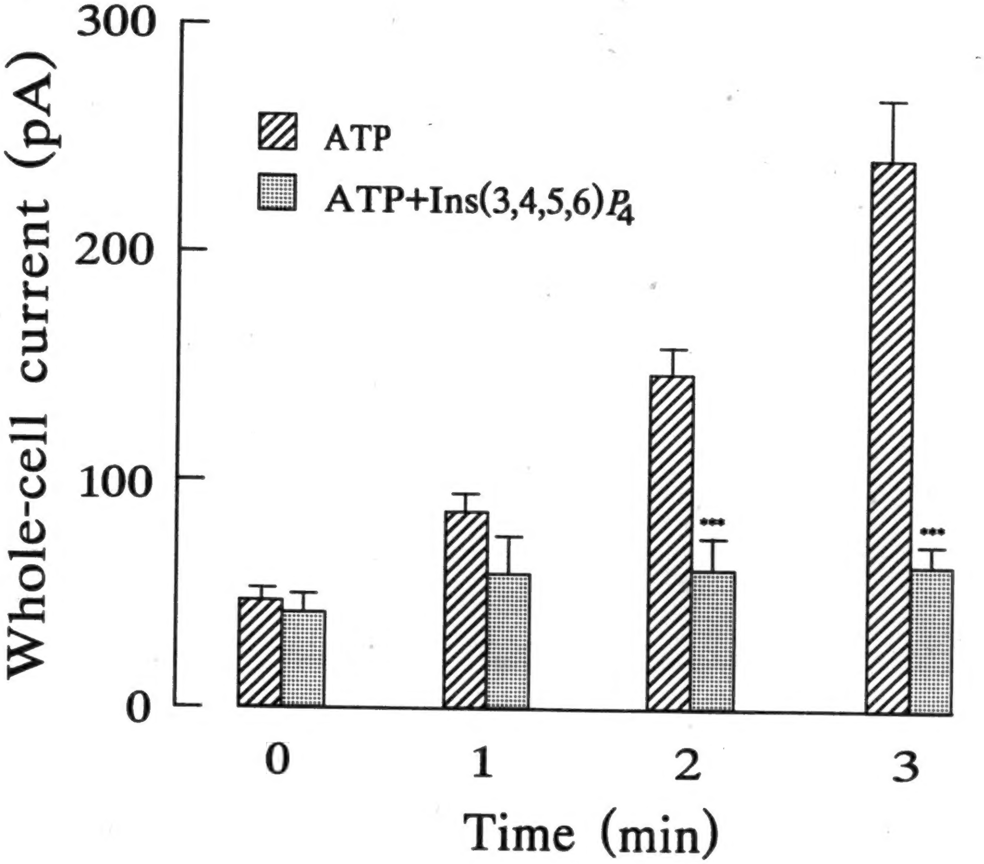

Figure 3.5.2. Development of ATP-activated Cl⁻ current with added intracellular Ins(3,4,5,6)P_4. The mean current (±S.E.) at +90 mV plotted over three minutes of the recording with 2 μM ATP applied to the bath solution, with and without 10 μM intracellular Ins(3,4,5,6)P_4 (*** $p \leq 0.001$ compared to the current recorded with 2 μM ATP alone). Mean current recorded with ATP plus 10 μM Ins(3,4,5,6)P_4 became significantly smaller than the current recorded with extracellular ATP alone by the second minute of recording. Number of experiments was between six and eight.

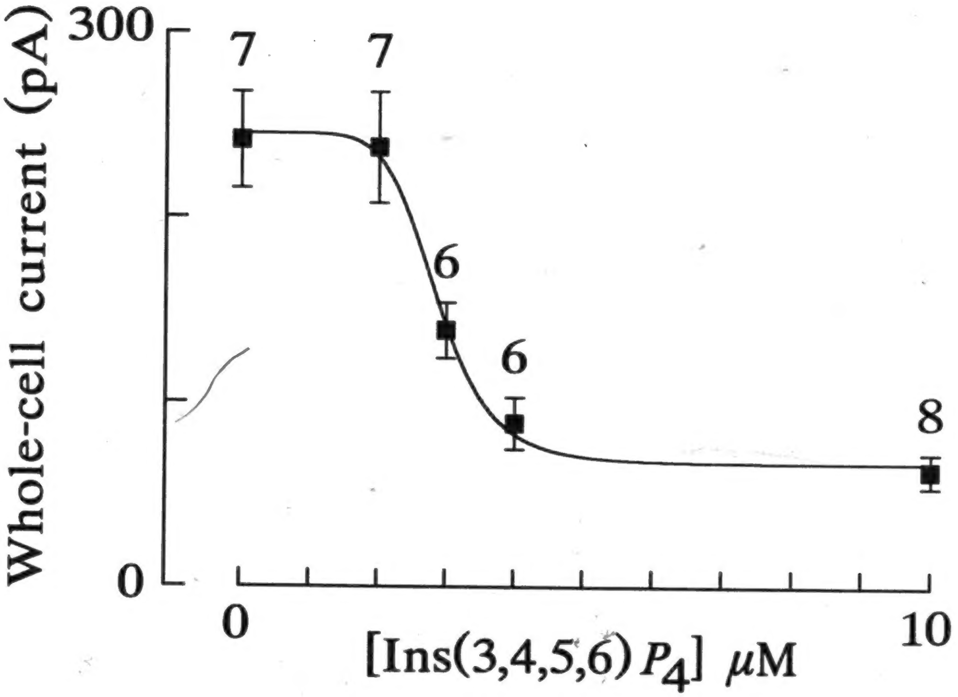

Figure 3.5.3. Dose-dependent inhibition of the ATP-activated Cl^- current by Ins(3,4,5,6)P_4. The dose-response curve for Ins(3,4,5,6)P_4 inhibition of Cl^- current. The numbers of experiments in each case are indicated directly above the plotted mean values. Data were fitted by Equation (1) with $I_{max}=177.8$ pA, $I_{base}=67.96$ pA, $K=2.87$ µM and $a=7.14$. The inhibitory action of Ins(3,4,5,6)P_4 on ATP-activated Cl^- current was sharply reduced as its concentration changed from 4 µM to 2 µM.

Ca^{2+} mobilization (Vajanaphanich *et al.*, 1994; Xie *et al.*, 1996). In addition, Ins(3,4,5,6)P_4 does not affect cellular calcium fluxes in other systems (DeLisle *et el.*,1994; Lu *et al.*, 1996). Nevertheless, we also investigated the effect of Ins(3,4,5,6)P_4upon Ca^{2+}-dependent Cl^- channels in CFPAC-1 cells when Ca^{2+} levels were elevated by a method that is entirely independent of signalling by ATP. In this case, Ca^{2+} was mobilized by 1 μM TG to inhibit the ATPase of the endoplasmic reticulum. In this experiment, Ins(3,4,5,6)P_4 still effectively blocked the Ca^{2+}-dependent Cl^- current (Fig. 3.5.4). The inhibitory effect of Ins(3,4,5,6)P_4 on TG-activated Cl^- current became significant ($p \leq 0.01$) within a minute and remained so ($p \leq 0.001$) for the rest of recording period (n=5-10).

Activation of surface PLC-linked receptors generates different InsP_4s as a result of the breakdown of Ins(1,4,5)P_3 via different metabolic pathways (Shears 1996). To test if the inhibition of ATP-activated Cl^- current by Ins(3,4,5,6)P_4 was specific to that particular polyphosphate, I examined the effects of three other isomers, Ins(1,3,4,5)P_4, Ins(1,4,5,6)P_4 and Ins(1,3,4,6)P_4 which are also generated from the same cycle (Fig. 1.6). Under the same recording conditions, none of the other InsP_4 isomers inhibited the ATP-induced Cl^- current at concentrations of 10 μM (Figs. 3.5.5, 3.5.6, & 3.5.7). Instead, all three potentiated the effect of ATP on the whole-cell current by varying amounts (Fig. 3.5.8). Nevertheless, the ATP-activated current measured in the presence of these InsP_4 isomers retained its normal

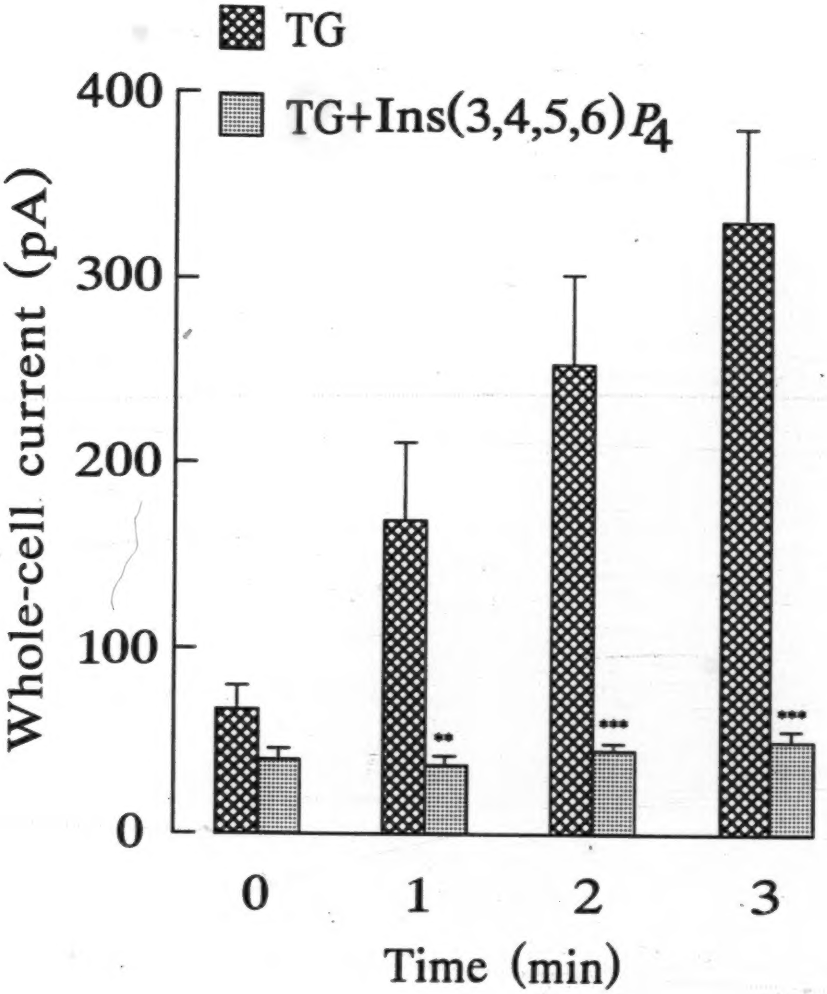

Figure 3.5.4. Ins(3,4,5,6)P_4 also inhibited a receptor-independent, Ca^{2+}-dependent whole-cell current. Thapsigargan was used to increase intracellular Ca^{2+} and therefore activate the Ca^{2+}-dependent Cl^- current without receptor stimulation. Mean current (±S.E.) at +90 mV with 1 μM TG or 1 μM TG plus 10 μM Ins(3,4,5,6)P_4, plotted during three minutes of recording (** $p \leq 0.01$, *** $p \leq 0.001$ compared to the current recorded with 1 μM TG alone). The inhibitory effect of Ins(3,4,5,6)P_4 became significant ($p \leq 0.01$) within a minute after the addition of TG, and remained so throughout the recording ($p \leq 0.001$). Number of experiments was between five and ten.

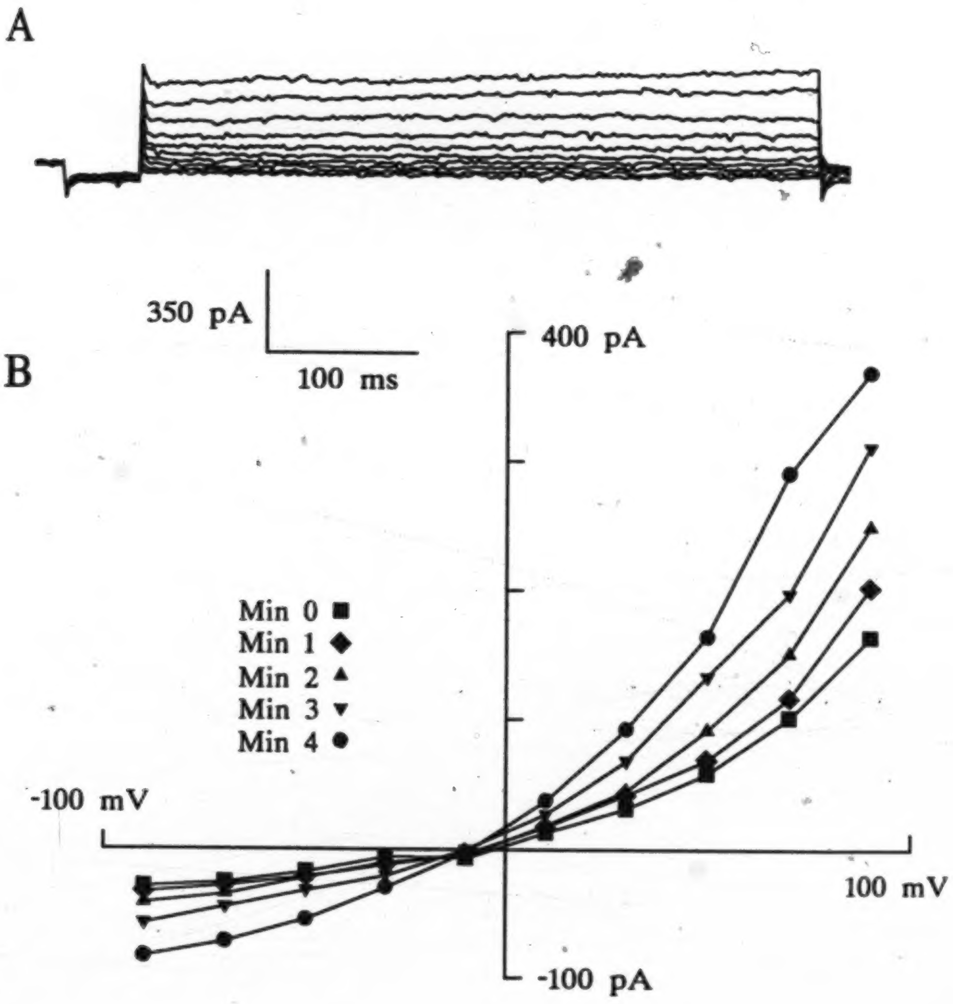

Figure 3.5.5. Typical ATP-activated whole-cell current with added intracellular Ins(1,4,5,6)P_4. (A) Actual current traces recorded four minutes after the application of 2 μM ATP to the bath solution, with 10 μM Ins(1,4,5,6)P_4 present in the pipet solution, (B) current-voltage relationships obtained from the same cell as in (A) as a function of time.

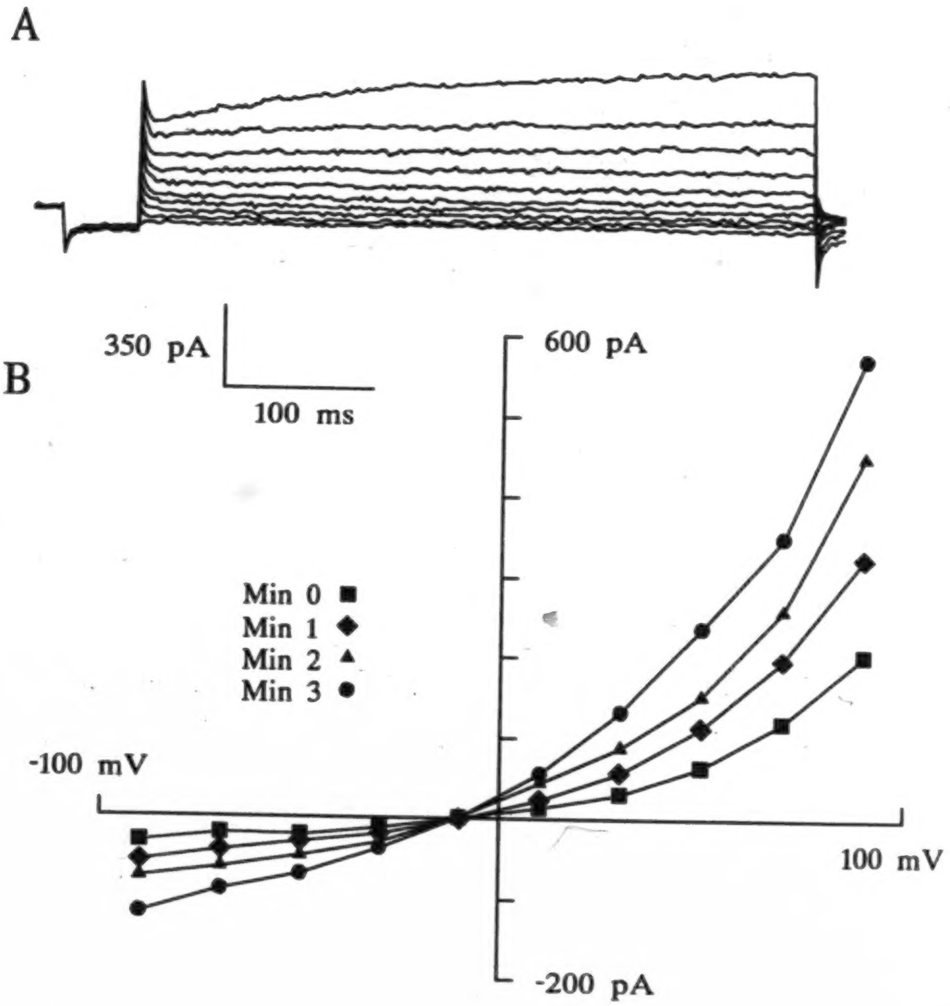

Figure 3.5.6. Typical ATP-activated whole-cell current with added intracellular Ins(1,3,4,5)P_4. (A) Actual current traces recorded three minutes after the application of 2 μM ATP to the bath solution, with 10 μM Ins(1,3,4,5,)P_4 being present in the pipet solution, (B) current-voltage relationships obtained from the same cell as in (A).

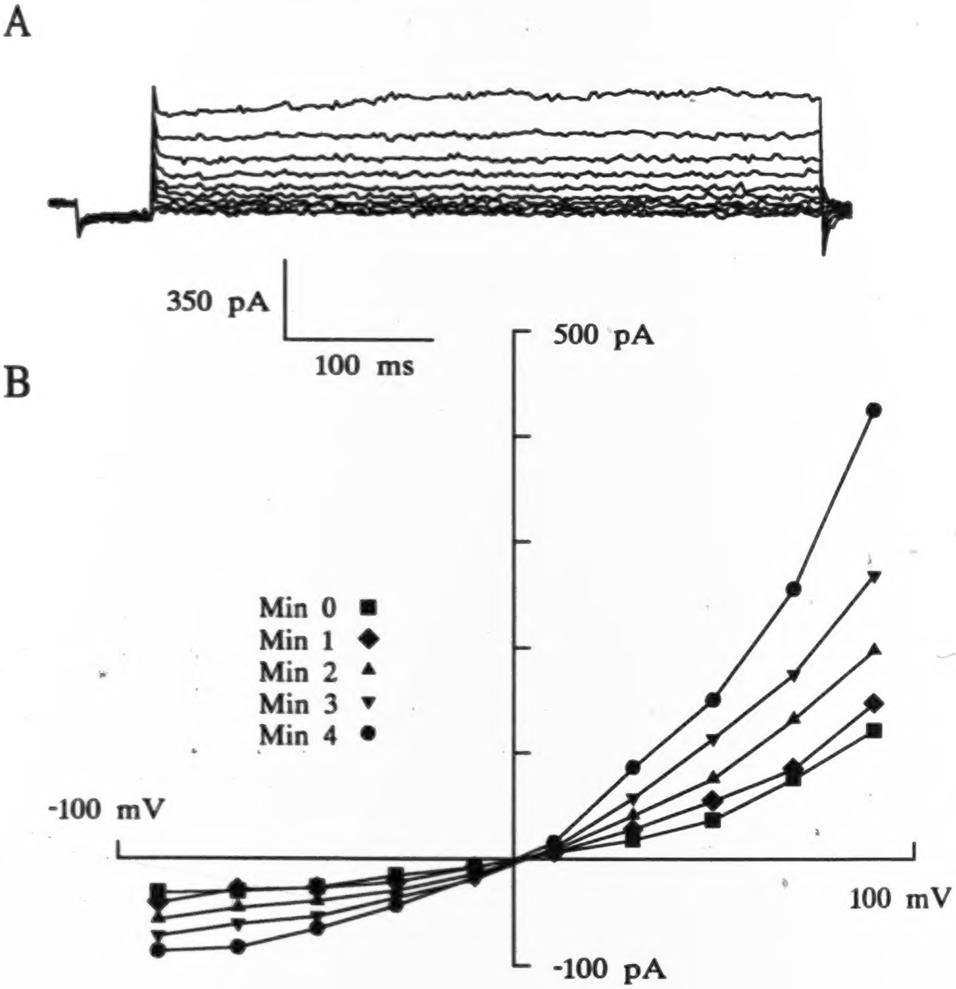

Figure 3.5.7. Typical ATP-activated whole-cell current with added intracellular Ins(1,3,4,6)P_4. (A) Actual current traces recorded four minutes after the application of 2 μM ATP to the bath solution, with 10 μM Ins(1,3,4,6,)P_4 present in the pipet solution, (B) current-voltage relationships obtained from the same cell as in (A).

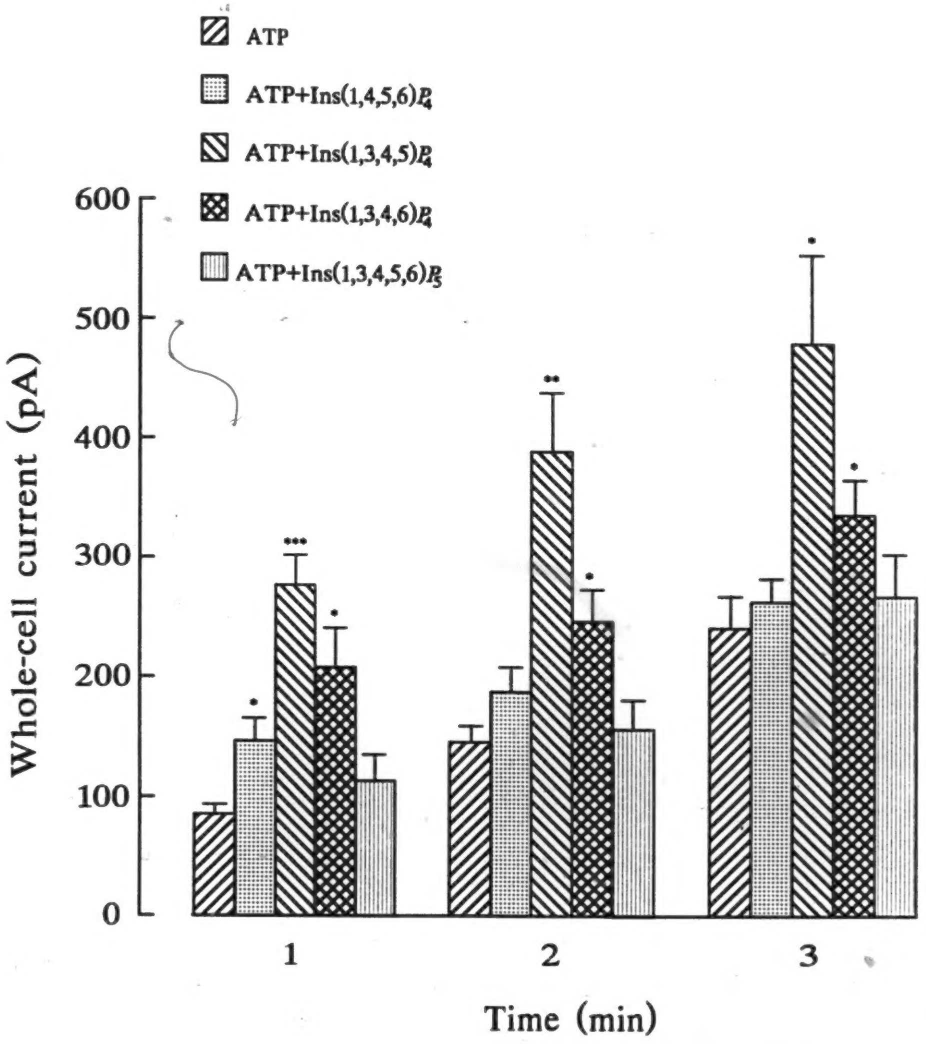

Figure 3.5.8. Development of the ATP-activated whole-cell current with different intracellular inositol phosphates. Mean ATP-activated current (±S.E.) at +90 mV with different intracellular inositol phosphates plotted over three minutes of the recordings ($*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$ compared to the whole-cell current with ATP alone at each minute). Number of experiments was between six and nineteen. Addition of Ins(1,3,4,5)P_4 or Ins(1,3,4,6)P_4 induced a whole cell current that was significantly greater than the current with ATP alone throughout the recording period. Ins(1,4,5,6)P_4 only significantly enhanced the effect of ATP during the first minute of recording, whereas Ins(1,3,4,5,6)P_5 had no effect on the whole-cell current.

characteristics, including the strong outward rectification and the slow activation at constant voltage (Figs. 3.5.5, 3.5.6, & 3.5.7). The effects of Ins(1,3,4,5)P_4 and Ins(1,3,4,6)P_4 remained significant throughout the recording period (Fig. 3.5.8). In contrast, Ins(1,4,5,6)P_4 elicited a transient elevation in current that was evident for only the first minute of recording (Fig. 3.5.8). The actions of Ins(1,3,4,5)P_4, Ins(1,4,5,6)P_4 and Ins(1,3,4,6)P_4 contrast strongly with that of Ins(3,4,5,6)P_4, which is the only InsP_4 isomer that inhibited whole-cell Cl^- current.

Stimulatory effects of Ins(1,3,4,5)P_4 and Ins(1,3,4,6)P_4 on whole-cell current were evidence even in the absence of ATP (Fig. 3.5.9). The mean current (±S.E.) recorded with Ins(1,3,4,5)P_4 ($n=19$) after two minutes recording was small compared to the current recorded in the presence of ATP, although this was still significantly ($p \leq 0.001$) greater than the control ($n=6$). Ins(1,3,4,6)P_4 also significantly elevated whole-cell current, but to an even lesser extent ($p \leq 0.05$, $n=17$). Ins(1,4,5,6)P_4 had no significant effect on the whole-cell current. Note that Ins(3,4,5,6)P_4 did not affect basal current in the absence of extracellular ATP (Fig. 3.5.9). Its effects are therefore highly specific to the Ca^{2+}-activated Cl^- current.

The InsP_4 isomers that we found to elevate ATP-activated Cl^- current, Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4 and Ins(1,4,5,6)P_4, have all been reported to mobilize Ca^{2+} by varying amounts (DeLisle *et el.*, 1994; Irvine 1992; Ivorra *et al.*, 1991; Lu *et al.*, 1996). It is therefore possible that these particular polyphosphates complement

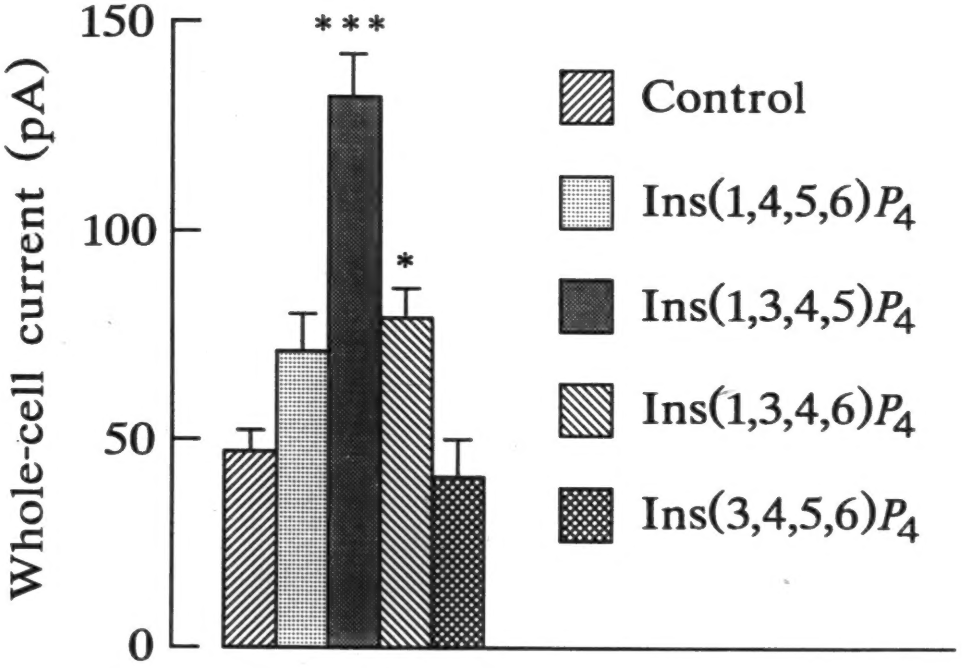

Figure 3.5.9. Effects of InsP_4 isomers on whole-cell current in the absence of extracellular ATP. The data represent mean currents (±S.E.) recorded at +90 mV in the absence of extracellular ATP after two minutes dialysis with pipet solutions containing 10 μM Ins(1,4,5,6)P_4 ($n=7$), Ins(1,3,4,5)P_4 ($n=19$), Ins(1,3,4,6)P_4 ($n=17$) or Ins(3,4,5,6)P_4 ($n=7$) (* $p \leq 0.05$, *** $p \leq 0.001$ compared to controls, $n=6$). Both Ins(1,3,4,5)P_4 and Ins(1,3,4,6)P_4 activated a significant whole-cell current even without the presence of ATP.

the action of ATP by augmenting the mobilization of Ca^{2+} by Ins(1,4,5)P_3. The Ca^{2+}-dependence of the stimulating effects of Ins(1,3,4,5)P_4 and Ins(1,3,4,6)P_4 on whole-cell current could be clearly demonstrated by diffusing them into cells under conditions where there could not be any Ca^{2+} mobilization. That is, intra- and extracellular free Ca^{2+} were reduced to approximately zero and the cells were not stimulated with extracellular ATP. Under these conditions, after two minutes of recording with 10 μM of either Ins(1,3,4,6)P_4 or Ins(1,3,4,5)P_4 in the pipet, the mean currents (±S.E.) were significantly smaller ($p \leq 0.001$) than the corresponding currents recorded under normal conditions (Fig. 3.5.10).

I next considered whether the enhancement of ATP-activated Cl^- current by Ins(1,3,4,6)P_4 and Ins(1,3,4,5)P_4 might modify the inhibitory actions of Ins(3,4,5,6)P_4. I therefore studied the effect of coinjecting 10 μM Ins(3,4,5,6)P_4 with 10 μM of either Ins(1,3,4,6)P_4 or Ins(1,3,4,5)P_4. Ins(3,4,5,6)P_4 was still effective in inhibiting the ATP-activated Cl^- current despite the presence of Ins(1,3,4,6)P_4 or Ins(1,3,4,5)P_4 and its inhibitory effect became significant ($p \leq 0.001$, n=7 for Ins(1,3,4,5)P_4; $p \leq 0.01$, n=6-10 for Ins(1,3,4,6)P_4) two minutes after the addition of ATP (Fig. 3.5.11). However, Ins(1,3,4,6)P_4, did slightly reduce the efficacy of Ins(3,4,5,6)P_4. This effect was most evident three minutes after purinergic activation, at which point Ins(3,4,5,6)P_4 alone reduced the absolute ATP-activated current by 178 pA (Fig. 3.5.2). In comparison, the current was reduced 118 pA by an equimolar mixture of Ins(1,3,4,6)P_4 plus Ins(3,4,5,6)P_4 (Fig. 3.5.11). That is, Ins(3,4,5,6)P_4 was only 30%

less effective as an inhibitor when equal concentration of Ins(1,3,4,6)P_4 was also present.

Ins(1,3,4,5,6)P_5 is both the precursor and the major metabolite of Ins(3,4,5,6)P_4 (Menniti *et al.*, 1990). I therefore examined the effects of 10 μM Ins(1,3,4,5,6)P_5 in the pipet on the whole-cell current. Ins(1,3,4,5,6)P_5 had no detectable effect on the unstimulated basal current (Fig. 3.5.12A), and when the same cells were then treated with ATP, typical receptor-dependent Cl^- currents developed (Figs. 3.5.12B & C), which is indicative of normal Cl^- channel activity. Unlike Ins(1,3,4,5)P_4 or Ins(1,3,4,6)P_4, Ins(1,3,4,5,6)P_5 did not significantly affect the ATP-activated Cl^- current throughout the recording period (Fig. 3.5.8).

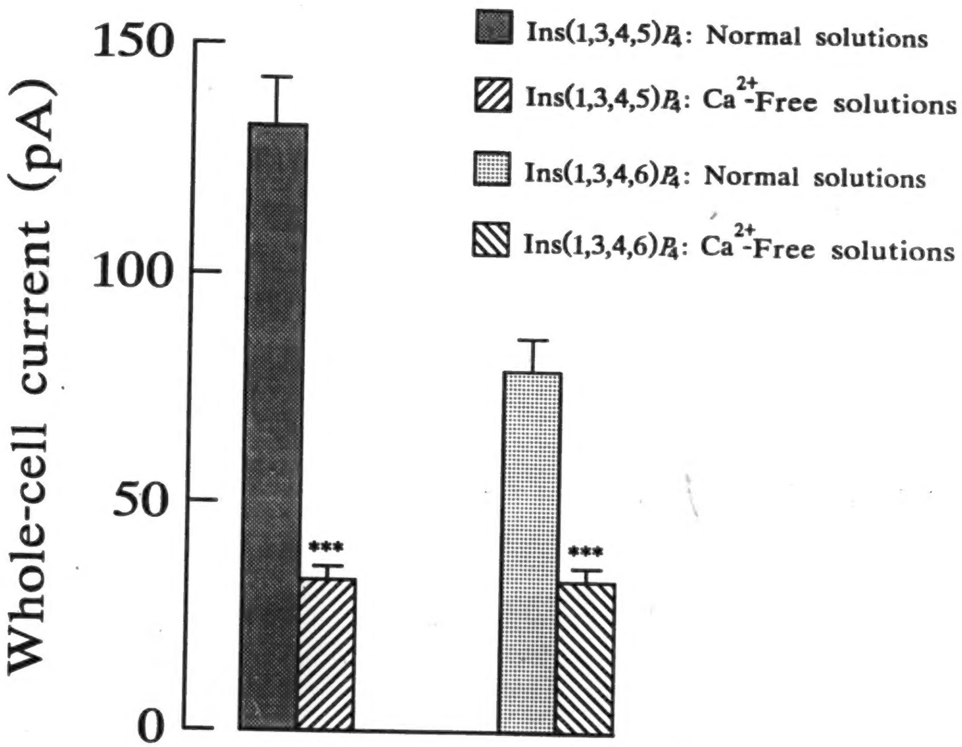

Figure 3.5.10. The stimulatory effects of Ins(1,3,4,5)P_4 and Ins(1,3,4,6)P_4 on whole-cell current were Ca^{2+}-dependent. Mean currents (±S.E.) recorded at +90 mV in the absence of extracellular ATP after two minutes of dialysis with 10 μM Ins(1,3,4,5)P_4 or Ins(1,3,4,6)P_4 in the normal or the zero-Ca^{2+} bath and pipet solutions (*** $p \leq 0.001$ compared to the current recorded with either Ins(1,3,4,5)P_4 or Ins(1,3,4,6)P_4 in the normal solutions). Number of experiments was between eight and nineteen.

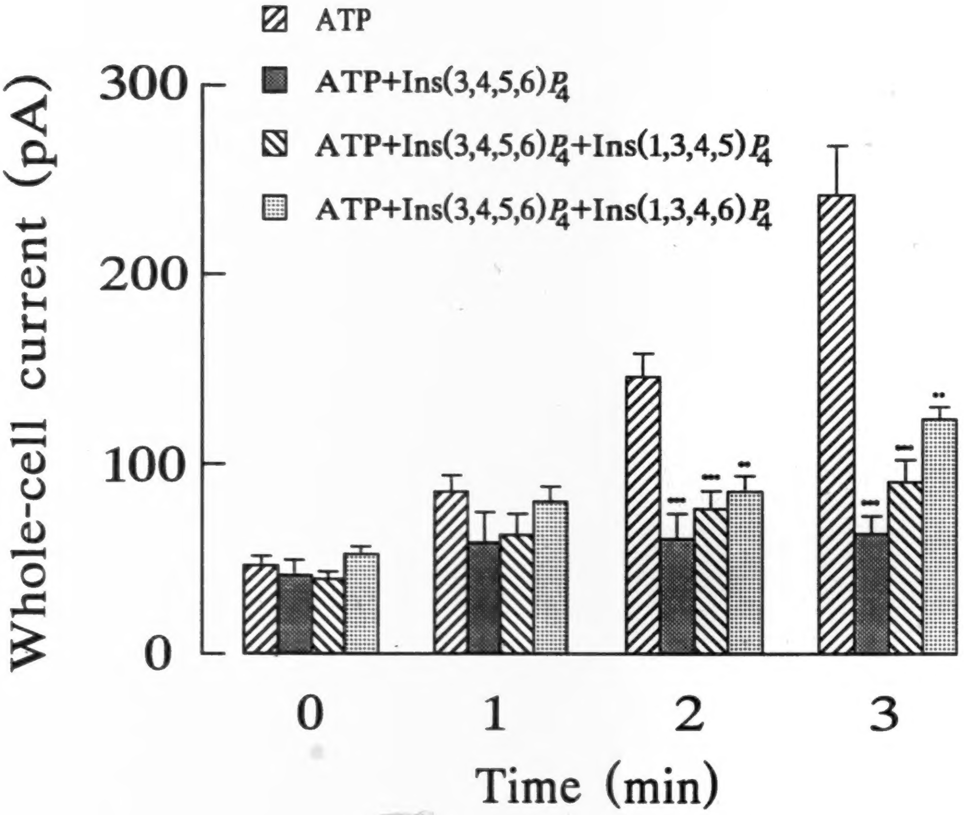

Figure 3.5.11. The inhibitory effect of Ins(3,4,5,6)P_4 was not affected by the stimulatory InsP_4s. Mean ATP-activated whole-cell current (±S.E.) recorded at +90 mV with the addition of 10 μM Ins(3,4,5,6)P_4 and 10 μM of either Ins(1,3,4,5)P_4 or Ins(1,3,4,6)P_4 in the pipet solution (** $p \leq 0.01$ and *** $p \leq 0.001$ compared to the current recorded with ATP alone). Number of experiments was between six and ten. Despite a small reduction in efficacy when coinjected with Ins(1,3,4,5)P_4 or Ins(1,3,4,6)P_4, Ins(3,4,5,6)P_4 could still inhibit a significant portion of the ATP-activated Ca^{2+}-dependent Cl^- current.

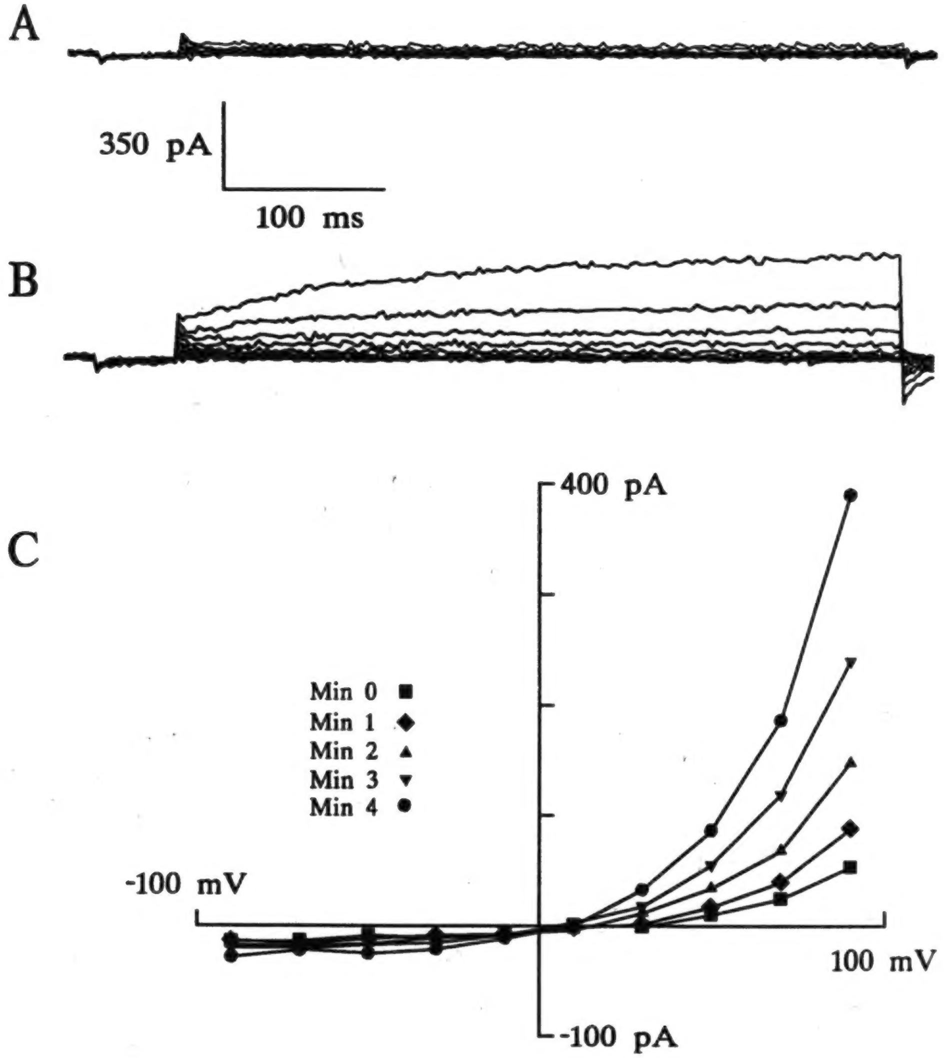

Figure 3.5.12. Intracellular Ins(1,3,4,5,6)P_5 did not affect the ATP-activated current. (A) Currents recorded with 10 μM Ins(1,3,4,5,6)P_5 and without ATP after two minutes. (B) currents recorded from the same cell, four minutes after addition of 2 μM extracellular ATP to the bath solution, and (C) current-voltage relationships obtained from the same cell. See Fig. 3.5.8 for the mean currents (±S.E.) recorded with Ins(1,3,4,5,6)P_5.

4. DISCUSSION

Chloride channels, like other groups of ion channels can be categorized in several different ways. Functional characterizations are usually based on the biophysical properties of channels (single-channel conductance, current-voltage relationship, kinetics properties etc.) and the regulatory mechanisms (ligand-gated, voltage-gated, calcium-gated etc.). For many channels these functional characterizations are now being followed by molecular characterization, as information about the amino-acid sequences and other chemical properties of the molecules become available. The work described in this thesis investigated two groups of epithelial Cl^- channels, the swelling-activated Cl^- channels of T84 cells and the Ca^{2+}-activated Cl^- channels of CFPAC-1 cells. In both cases, information was obtained about the biophysical properties and the regulation of the channels involved. These findings will be discussed primarily in terms of their implications for the ion transport functions of epithelia, and their place in the broader context of current knowledge in this area.

4.1 Cell volume regulation in T84 cells

The swelling-activated current observed here had a similar amplitude and time course to the current reported before in T84 cells (Worrell *et al.*, 1989) and in T-lymphocytes (Lewis *et al.*, 1993). Cell swelling occurred within one minute of whole-cell formation, but the swelling-activated current developed slowly over the recording period. It was also apparently carried by chloride ions, as indicated by the reversal potential and DIDS sensitivity. However, the electrical properties were quite different to previous observations. The current observed here was strongly inwardly rectifying and there was no indication of significant activation or inactivation with time after voltage steps, even with steps from -100 mV to 90 mV (Fig. 3.1.1) as reported in other studies (Kubo & Okada, 1991; Paulmichl *et al.*, 1992; Thiemann *et al.*, 1992; Valverde *et al.*, 1992; Worrell *et al.*, 1989). Inwardly rectifying chloride channels have been reported before in epithelia and mouse mandibular gland cells, although with much larger conductances than found here (Duszyk *et al.*, 1991; Hanrahan *et al.*, 1985; Komwatana *et al*, 1994).

Another important difference was the estimated single-channel conductance of the channels responsible for the swelling-activated current. The earlier measurements on T84 cells suggested a conductance of ~50-75 pS. These estimates were based on the outward rectification of the current and the amplitudes of discrete shifts in whole-cell currents resembling single-channel events (Worrell *et al.*, 1989), as well as cell-attached single-channel recordings from swollen cells (Solc & Wine 1991). However, there were similarities between the channels activated by volume changes and by voltage, and some of the channel recordings assumed that both voltage and swelling activated the same set of channels. Recent noise analysis studies with a variety of cells have also shown swelling-activated Cl^- channels which were well below 5 pS (Lewis *et al.*, 1993; Nilius *et al.*, 1994). Our estimate of 0.2 pS is in close agreement with the value of 0.2 and 0.4 pS obtained by noise analysis in C3H 10T1/2 and COS-1 cells, and is compatible with the finding that swelling-activated chloride channels were too small to be resolved in HT_{29} cells (Kunzelmann *et al.*, 1992; Nilius *et al.*, 1994).

If the current is carried by such low-conductance channels, then the required channel density is high. The analysis gave an estimate of ~90,000 per cell. Using an average cell diameter of 20 μm gives a density of ~70 channels/μm^2. Although quite high, this figure is within the known range of ion channel densities and much lower than the estimated density of 10,000 channels/μm^2 estimated for acetylcholine channels at the motor endplate (Hille 1992). Verification of the existence of such low-conductance and densely packed channels by single-channel recording would be difficult. However, the estimates obtained here from noise analysis were consistent, and noise analysis has previously predicted the presence of low-conductance channels in epithelia that were later found by single-channel analysis (Duszyk *et al.*, 1992; Wilk-Blaszczak *et al.*, 1992). It is therefore likely that the swelling-activated channels represent the most common ion channels in the cell membrane and a significant fraction of the total membrane proteins.

The cause of the double Lorentzian characteristic of the current noise is not yet known. In general, a double Lorentzian function may be due to the presence of two ion channels or to a single channel which spends significant amounts of time in several different open or closed states. The variance of the low frequency component did not increase during the development of the swelling-activated current, its corner frequency was stable, and its mean amplitude after 5 minutes (0.55 pA^2) was similar to the time-invariant component of the total current variance (σ_0^2=0.43 pA^2). These data could mean that the low frequency component was due to a population of channels that were partially open at the start of each experiment and remained unaffected by the development of the swelling-activated current.

However, total membrane current at the start of each experiment was near zero (Fig. 3.1.3), so it seems more likely that only one population of channels with complex kinetics was responsible for the swelling-activated current. If the high frequency component of the Lorentzian were due to a separate group of channels, the 25% decrease in its corner frequency (76 to 60 Hz) would correspond to an increase in mean open time from 2.1 to 2.7 ms. Alternatively, if the two Lorentzian components were due to a single group of channels with more complex kinetics, their relative changes in amplitude and corner frequency would represent shifts in the occupancy of channel states, but still include an increase in total channel open time.

Earlier work showed that the treatment of larger epithelial chloride channels by the stilbene derivatives DNDS (Singh *et al.*, 1991) and SITS (Nelson *et al.*, 1984) caused a flickery block that was associated with reductions in channel open times.

Although DIDS also belongs to this class of Cl$^-$ channel blockers, no increases in total current variance or Lorentzian noise during the experiments with DIDS blockade was observed. The overall variance with DIDS was about 0.5 pA^2, which is close to the background variance, suggesting that flickery block was not evident.

One difference between these experiments and some other measurements of swelling-activated currents was the lack of intracellular ATP dependence (Best *et al.* 1996; Gill *et al.* 1992; Lewis *et al.*, 1993; Worrell *et al.*, 1989). It seems possible that ATP is not essential for producing the swelling-activated current (Gschwentner *et al.*, 1996; Solc & Wine 1991), but can stimulate the appearance of a separate current with a similar time course but different electrical characteristics.

Nucleotides have complex relationships with epithelial ion channels. In addition to their role in phosphorylation, nucleotides activate the CFTR protein by direct binding (Travis *et al.*, 1993). ATP has also been reported to activate a low-conductance chloride channel via a membrane-bound cGMP-dependent protein kinase, that was not related to cell swelling (Lin *et al.*, 1992). Recent studies have suggested that the 50 pS outwardly rectifying chloride channels can be activated by surface purinergic receptors via a Ca^{2+}-independent pathway (Schwiebert *et al.*, 1995; Stutts *et al.*, 1992). Inclusion of ATP in recording solutions would then be likely to cross-activate other conductances as well as the swelling-activated Cl$^-$ current. Here, the discrete membrane fluctuations, characteristic of rectifying channels, were never seen in cells without ATP in the pipet, but the swelling-activated current appeared reliably without ATP and produced noise that was well-fitted by a channel conductance of about 0.2 pS.

In terms of biophysical properties, the swelling-activated Cl^- current resembled the ClC-2-associated but not the Pgp- or the I_{Cln}-associated swelling-activated currents. Both this current and the ClC-2-associated current exhibited inward rectification and ATP-insensitivity, whereas the Pgp-associated current showed both outward rectification and intracellular ATP dependence (Thiemann *et al.*, 1992; Valverde et al, 1992). I_{Cln}-associated swelling-activated current is also outwardly rectifying, insensitive to intracellular Ca^{2+} and is blocked by external ATP (Paulmichl *et al.*, 1992).

The ClC-2 gene product has been shown to be expressed in a variety of cells including T84 cells (Thiemann *et al.*, 1992). However, the ClC-2-associated swelling-activated current also shows time-dependent activation and insensitivity to DIDS. These characteristics of the ClC-2-associated current are not in total agreement with the present observations (Thiemann *et al.*, 1992). Other Cl^- conductances have similarities to ClC-2 based on biophysical properties (Komwatana *et al.*, 1994) and amino acid sequence (Malinowska *et al.*, 1995), but also have some differences, such as blocker sensitivity or biophysical properties. Nevertheless, the swelling-activated 0.2 pS Cl^- channel observed here seems more closely related to the

ClC-2 Cl^- channel than the Pgp- and the I_{Cln}-associated conductances, in terms of its biophysical properties.

This work demonstrated that T84 cells express a low-conductance Cl^- channel which is activated by hypo-osmotic stress. The exact gating mechanism(s) for this low-conductance Cl^- channel were not examined in detail. However, a few possible mechanisms for channel activation can be deduced from the recording conditions. First, a Ca^{2+}-dependent pathway for channel activation seems unlikely due to the high EGTA content in the pipet solution. Second, voltage-dependent activation of this Cl^- channel is unlikely due to the lack of current development under non-swelling conditions. However, a phosphorylation-dependent mechanism for channel activation cannot be completely excluded. In spite of the lack of exogenous ATP in the pipet solution, it is possible that sufficient cellular ATP was available for phosphorylation, even after cell dialysis. Nevertheless, this Cl^- channel had very low conductance and was expressed abundantly in epithelial cells. An understanding of its gating properties could provide an alternative means of increasing Cl^- conductance, and thus be of possible value in CF treatment.

4.2 Surface P_2 purinoceptors in CFPAC-1 cells

Like many other epithelial cells, CFPAC-1 cells showed a sensitivity to extracellular nucleotides, responding with an increase in $[Ca^{2+}]_i$. The biphasic response to ATP was in agreement with a previous study in CFPAC-1 cells (Klär *et al.*, 1993) and other studies with different cell types (Nilius *et al.*, 1995b; Stutts *et al.*, 1994). The initial transient increase in $[Ca^{2+}]_i$ by ATP was most likely due to mobilization from intracellular Ca^{2+} stores. This was supported by two pieces of evidence: (1) replacement of the normal bath solution with Ca^{2+}-free solution did not affect the transient peak $[Ca^{2+}]_i$, and (2) depletion of intracellular Ca^{2+} stores with TG totally abolished the ATP-induced response.

It is probable that ATP mobilized Ca^{2+} in CFPAC-1 cells via an Ins(1,4,5)P_3-dependent pathway as a result of PLC activation. It has been shown that G-protein coupled P_2 purinergic receptors like P_{2Y} and P_{2U} are commonly linked to a PLC-dependent pathway (Chen *et al.*, 1996; Cockcroft & Stutchfield 1989; Dubyak *et al.*, 1988; Siddiqui & Exton 1992; Zhang *et al.*, 1995). A recent study of human glomerular epithelial cells has demonstrated a parallel increase in Ins(1,4,5)P_3 upon ATP stimulation (Pavenstädt *et al.*, 1992).

The second slow, or plateau, phase of the ATP-induced response in CFPAC-1 cells probably resulted from agonist-induced Ca^{2+} entry. This finding has also been observed in other epithelial cells (Ikeda *et al.*, 1995). However, the exact mechanism(s) for Ca^{2+} entry remains controversial. Entry of extracellular Ca^{2+} during the plateau phase was probably due to inositol phosphates from the breakdown of Ins(1,4,5)P_3, as well as Ins(1,4,5)P_3 itself (Bird *et al.*, 1991). One such inositol phosphate would be Ins(1,3,4,5)P_4, which has been reported to stimulate Ca^{2+} entry via a second messenger-activated plasma membrane Ca^{2+} channel (Lückhoff & Clapham 1992) or other unidentified pathways (Hashii *et al.*, 1993; Irvine 1992). However, depletion of intracellular Ca^{2+} stores has been reported to activate Ca^{2+} entry, which is Ins(1,4,5)P_3- and Ins(1,3,4,5)P_4-independent (Hoth & Penner 1992). The other inositol phosphates, Ins(4,5)P_2, Ins(1,4,6)P_3, Ins(2,4,5)P_3, Ins(1,2,4,5)P_4 and Ins(1,3,4,6)P_4, have also been demonstrated to induce Ca^{2+} entry when injected into *Xenopus* oocytes (DeLisle *et al.*, 1995).

The ATP-stimulated increase in $[Ca^{2+}]_i$ decayed slowly towards the baseline despite the continuous presence of ATP in CFPAC-1 cells. This slow decay in $[Ca^{2+}]_i$ could be the result of mitochondrial reuptake, activation of a Ca^{2+} pump or other Ca^{2+} transport mechanisms in the plasma membrane, or simply exhaustion of intracellular Ca^{2+} pools. These findings were not consistent with an earlier study using the same cell type, showing that the plateau response remained elevated for over ten minutes (Klär *et al.*, 1993).

The ability of UTP to elicit a similar Ca^{2+} response indicated the possible expression of a P_{2U} receptor in CFPAC-1 cells, based on the known selectivity sequence of these receptors for different nucleotides (Dubyak & El-Moatassim 1993). Only the P_{2U} subtype of purinergic receptors has similar sensitivities to both extracellular ATP and UTP. A previous study has also reported UTP sensitivity on

CFPAC-1 cells (Galietta *et al.*, 1994). Despite similar expression of P_{2U} receptors in a variety of cell types (Dubyak & El-Moatassim 1993), the possible coexpression of P_{2Y} and P_{2U} receptors could not be excluded in this case. Coexpression of P_{2Y} and P_{2U} receptors has been reported in kidney epithelia (Zegarra-Moran *et al.*, 1995). However, the expression of P1 receptors in CFPAC-1 cells was not tested. It is possible that part of the ATP-induced Ca^{2+} response was due to P_1 purinoceptor activation as a result of the rapid breakdown of extracellular ATP to adenosine by ecto-ATPase or ectonucleotidase (Dubyak & El-Moatassim 1993). Expression of P_1 purinoceptors has been demonstrated in airway (Chao *et al.*, 1994; Lazarowski *et al.*, 1992). Nevertheless, CFPAC-1 cells express functional P_{2U} purinoceptors, and therefore, are extremely useful for studying how extracellular nucleotides regulate ion transport in CF epithelial cells.

4.3 Regulation of the purinoceptor-activated, Ca^{2+}-dependent Cl^- current in CFPAC-1 cells

Surface purinoceptors are widely expressed in many different cell types and activation of these receptors has been shown to stimulate Cl^- current (Clarke & Boucher 1992; Mason *et al.*, 1991; Stutts *et al.*, 1994). In the previous section, I confirmed the expression of a functional surface P_2 purinoceptor in CFPAC-1 cells. Here, electrophysiological data confirm earlier reports (Galietta *et al.*, 1994; Warth & Greger 1993) that activation of purinergic receptors by ATP stimulates a Cl^- current in CFPAC-1 cells. These conclusions were based on two pieces of evidence:

(1) the ATP-activated current in CFPAC-1 cells always reversed near the Cl^- equilibrium potential, and (2) when the Cl^- channel blocker, DIDS was included in the bath solution, ATP was no longer able to induce the outward rectifying current.

Under the present experimental conditions, extracellular ATP activated a Cl^- current in CFPAC-1 cells that was similar to those of other epithelial cells in its temporal response to ATP, and in its outward rectification (Fig. 3.4.1) (Cliff & Frizzell 1990; Stutts *et al.*, 1994). These biophysical properties of the ATP-stimulated whole-cell current indicate that the chloride channels causing it have strong voltage-dependent activation. The slow activation at constant voltage could be due to an increase in the number of channels being activated and/or an increase in open probability with time. However, it is not possible to directly compare these data with earlier studies of CFPAC-1 cells (Galietta *et al.*, 1994; Warth & Greger 1993), because these reports did not include whole-cell current data.

Although recent studies in CF airway epithelial cells have indicated that ATP activates both Ca^{2+}-sensitive and Ca^{2+}-insensitive Cl^- conductances (Schwiebert *et al.*, 1995; Stutts *et al.*, 1994), the Ca^{2+}-dependence of the present ATP-activated current was supported by two lines of evidence: (1) there was no effect of ATP on whole-cell current under zero-Ca^{2+} conditions (Fig. 3.4.4). (2) when intracellular Ca^{2+} concentration was elevated by inhibiting the Ca^{2+}-ATPase of endoplasmic reticulum with TG, the ensuing activated whole-cell current was electrophysiologically similar to that induced by ATP (Fig. 3.4.5).

The contribution of K^+ current to the overall ATP-activated whole-cell current was minimal in these experiments (Fig. 3.4.3). Addition of DIDS blocked almost all of the Cl^- current, and the remaining current was likely to be a K^+ current based on the shift in the reversal potential. It is very likely that this small K^+ current in CFPAC-1 cells resulted from activation of an epithelial Ca^{2+}-dependent K^+ channel. This is supported by the zero-Ca^{2+} experiments in which ATP could induce neither the Cl^- current nor this "residual" K^+ current. A Ca^{2+}-dependent K^+ channel has also been observed in other epithelial cells as well as CFPAC-1 cells (Galietta *et al.*, 1994; Nilius *et al.*, 1995b). However, the ATP-stimulated K^+ currents reported in these studies were more prominent. These differences between the present and the previous studies in CFPAC-1 cells can not be easily explained, but might be due to differences in culture conditions and subsequent protein expression.

Ins(1,4,5)P_3 is a well established signalling molecule that mobilizes cellular Ca^{2+} (Berridge & Irvine 1989), and there is a large body of evidence that Ins(1,3,4,5)P_4 might also contribute to overall Ca^{2+} signalling (Irvine 1992). However, the complexities of the inositol phosphate metabolic pathway suggest that some additional inositol polyphosphates could also have important functions. In particular, it was pointed out that the characteristics of the receptor-activated turnover of Ins(3,4,5,6)P_4 were indicative of it being an intracellular signal (Menniti *et al.*, 1990).

Subsequent studies indicated that Ins(3,4,5,6)P_4 inhibited Ca^{2+}-dependent chloride current in T84 colonic epithelial cells (Vajanaphanich *et al.*, 1994; Xie *et al.*, 1996).

The experiments described here were initially designed to test if Cl^- channels in the CFPAC-1 cell line are also regulated by Ins(3,4,5,6)P_4. This cell type was chosen for two main reasons: (1) they lack the cAMP-activated Cl^- current that is commonly found in epithelial cells, making it easier to isolate the Ca^{2+}-activated Cl^- current that is also prominent in epithelia, (2) CFPAC-1 cells represent a completely different epithelial cell type to the T84 colonic epithelial cell line that was used in the earlier studies (Vajanaphanich *et al.*, 1994; Xie *et al.*, 1996).

The experiments demonstrated that Ins(3,4,5,6)P_4 specifically inhibited receptor-activated, Ca^{2+}-dependent Cl^- current in CFPAC-1 cells (Figs. 3.5.1, 3.5.2 & 3.5.9) at physiologically relevant concentrations. For example, there was no significant effect on Cl^- current by 1-2 μM Ins(3,4,5,6)P_4 (Fig. 3.5.3), which is the concentration that occurs in resting cells (Vajanaphanich *et al.*, 1994). Inhibition of the Cl^- current by Ins(3,4,5,6)P_4 followed a simple dose-response relationship (Equation 1), with an apparent equilibrium constant of 2.86 μM. The rather high cooperativity value of 7.14, obtained by fitting the data in Fig. 3.5.3, suggests that several molecules of Ins(3,4,5,6)P_4 may need to react simultaneously to close the Cl^- channels. In any case, it is clear that the Cl^- current *in vivo* will be very sensitive to quite small changes in Ins(3,4,5,6)P_4 levels. The cellular concentrations of

$Ins(3,4,5,6)P_4$ that prevail during prolonged PLC activation (>3 μM, Vajanaphanich *et al.*, 1994) would be expected, according to these results (Fig. 3.5.3), to substantially inhibit the Cl^- current.

Several independent studies have shown that $Ins(3,4,5,6)P_4$ does not affect the process of Ca^{2+} mobilization (DeLisle *et el.*, 1994; Lu *et al.*, 1996, Vajanaphanich *et al.*, 1994). Thus, the mechanism by which $Ins(3,4,5,6)P_4$ inhibits Cl^- current in CFPAC-1 cells is more likely to reflect inhibition of the appropriate Cl^- channels, rather than block of a receptor-initiated Ca^{2+} signal. This conclusion is strengthened by the finding that $Ins(3,4,5,6)P_4$ could also inhibit the TG-activated Cl^- current effectively, since TG mobilizes Ca^{2+} by a mechanism that is independent of receptor-activation.

Some previous electrophysiological studies using the perforated patch clamp technique have reported that there is desensitization of receptor-dependent, PLC-mediated activation of Cl^- current (Galietta *et al.*, 1994; Morris *et al.*, 1990). Desensitization also seems to have been observed in some experiments with epithelial layers, in which receptor-activated, PLC-dependent Cl^- efflux was found to be transient rather than sustained, even in the continued presence of agonist-mediated PLC activity (Jiang *et al.*, 1993; Vajanaphanich *et al.*, 1994). The present data indicate that the elevations in levels of $Ins(3,4,5,6)P_4$ that accompany PLC activation (Menniti *et al.*, 1990) could contribute to this proposed desensitization process. The relatively slow metabolism of Ins(3,4,5,6)P_4 inside cells (Vajanaphanich *et al.*, 1994) is consistent with the proposed nature of this desensitization (Galietta *et al.*, 1994). Note that this phenomenon is less-readily detected during analysis of receptor-activated events by whole-cell patch clamp recording, because this procedure will dilute both the Ins(1,3,4,5,6)P_5 precursor pool, and the Ins(3,4,5,6)P_4 catabolic product.

The inhibition of Cl^- current by Ins(3,4,5,6)P_4 was also highly specific. None of the other InsP_4s, Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4, Ins(1,4,5,6)P_4 which are also generated after receptor activation, or Ins(1,3,4,5,6)P_5 had any inhibitory effects on Ca^{2+}-dependent Cl^- current. The absence of any effect by Ins(1,3,4,5,6)P_5 is particularly significant because this is the immediate precursor of Ins(3,4,5,6)P_4 (Menniti *et al.*, 1990). The lack of effect of Ins(1,3,4,5,6)P_5 on the Ca^{2+}-dependent Cl^- current can not be explained simply by the degradation of Ins(1,3,4,5,6)P_5. The metabolic flux from Ins(1,3,4,5,6)P_5 to Ins(3,4,5,6)P_4 upon receptor activation is a relatively slow (>10 mins) process (Vajanaphanich *et al.*, 1994) and is absolutely dependent upon intracellular ATP (Oliver *et al.*, 1992), which was absent from the pipet solution.

In contrast to the inhibitory effect of Ins(3,4,5,6)P_4 on Ca^{2+}-dependent Cl^- current, the whole-cell current was further increased by either Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4 or Ins(1,4,5,6)P_4 to varying degrees following cell stimulation by ATP. It is possible that these three polyphosphates potentiated the effects of ATP by enhancing Ca^{2+} mobilization. Consistent with this idea, all three of these InsP_4 isomers have previously been reported to activate Ca^{2+}-dependent Cl^- current when they were separately injected into *Xenopus* oocytes (DeLisle *et el.*, 1994). Furthermore, others have reported that Ins(1,3,4,5)P_4 and Ins(1,3,4,6)P_4 mobilize Ca^{2+} from the Ins(1,4,5)P_3-releasable pool (DeLisle *et el.*, 1994; Ivorra *et al.*, 1991) and Ins(1,3,4,5)P_4 might stimulate Ca^{2+} entry or might be dephosphorylated to Ins(1,4,5)P_3 by 3-phosphatase activity (Irvine 1992). The effect of Ins(1,3,4,6)P_4 on Cl^- current can be explained by its potential role in Ca^{2+} entry (DeLisle *et el.*, 1994). However, it should be noted that there has been considerable controversy and debate for many years on the relationship between Ins(1,3,4,5)P_4 and Ca^{2+} mobilization (Irvine 1992; Morris *et al.*, 1987). This issue was not directly pertinent to the present studies, and was not pursued it in detail, but it is clear in this case that the stimulatory effect of Ins(1,3,4,6)P_4 and Ins(1,3,4,5)P_4 were closely linked to Ca^{2+} availability (Fig. 3.5.10). However, the stimulatory effect of these InsP_4 isomers was not observed in T84 cells (Xie *et al.*, 1996). Nonetheless, the important point here is that of all the isomers tested, only Ins(3,4,5,6)P_4 inhibited ATP-activated Cl^- current. Therefore, the actions of Ins(3,4,5,6)P_4 are very specific.

It is known that Ins(3,4,5,6)P_4 is only one of the several InsP_4 isomers to increase in concentration to varying degrees upon agonist stimulation (Vajanaphanich *et al.*, 1994). It is therefore important to consider the possibility of interactions between these various InsP_4 isomers. The coinjection data showed that Ins(3,4,5,6)P_4

blocked the Ins(1,3,4,5)P_4-mediated enhancement of ATP-activated Cl^- current. While an equimolar dose of Ins(1,3,4,6)P_4 reduced the efficacy of Ins(3,4,5,6)P_4 as an inhibitor by 30%, a smaller effect would be anticipated in receptor-activated intact cells, where levels of Ins(3,4,5,6)P_4 are up to severalfold in excess of levels of Ins(1,3,4,6)P_4 (Menniti *et al.*, 1990; Vajanaphanich *et al.*, 1994). Thus, the present data indicate that under normal physiological conditions, where mixtures of different InsP_4 isomers are elevated by agonists, Ins(3,4,5,6)P_4 would be still an effective regulator of Cl^- secretion.

These new data on CFPAC-1 cells consolidate and advance previous work with the T84 colonic epithelial cell line (Vajanaphanich *et al.*, 1994; Xie *et al.*, 1996) on several fronts. First, it was possible to study the regulation of Ca^{2+}-dependent Cl^- current independently of any possible interaction with cAMP-regulated channels. Second, by demonstrating a direct signalling effect of Ins(3,4,5,6)P_4 in a different epithelial cell type we can now anticipate that this represents a more general physiological phenomenon. Third, these data indicate that Ins(3,4,5,6)P_4 is an effective inhibitor of Ca^{2+}-dependent Cl^- current irrespective of whether it is activated by PLC-linked receptor-occupation or by TG. Fourth, the dose/response curve demonstrates that CFPAC-1 cells are particularly sensitive to Ins(3,4,5,6)P_4. Finally, these data provide the first direct evidence that Ins(3,4,5,6)P_4 is a potent negative signalling molecule under physiological conditions in which stimulatory InsP_4s are also generated from receptor activation.

5. CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions

Apical Cl^- channels represent a major component of transepithelial Cl^- transport. The defective cAMP-regulated Cl^- conductance in CF causes many clinical problems such as pancreatic deficiency and deterioration of pulmonary functions. Treatment for CF has been concentrated on gene therapy, as well as using pharmacological means to stimulate alternative Cl^- conductances to compensate for the loss of the cAMP-regulated Cl^- conductance. The main purpose of this thesis was to study the regulation of some alternative Cl^- conductances, the swelling-activated and receptor-mediated, Ca^{2+}-dependent Cl^- currents for their potential use in CF treatment.

The two Cl^- conductances observed here exhibited different biophysical properties and regulatory mechanisms. The high estimated numbers of swelling-activated Cl^- channel in T84 cells suggest that they are commonly expressed in epithelial cells. The abundance of this swelling-activated Cl^- channel could be of potential benefit in CF treatment. Pharmacological modulation of these Cl^- channels might provide a means to enhance Cl^- secretion and therefore minimizing the clinical manifestations of CF.

Extracellular nucleotides have been proposed as a CF treatment. In this study, a functional purinergic receptor was identified in CFPAC-1 cells. Activation of this purinergic receptor induced a Ca^{2+}-dependent Cl^- current. However, the use of this pathway for CF treatment was shown to be limited. It was demonstrated for the first time in CF epithelial cells that one of the byproducts of the activation of PLC-linked receptors, Ins(3,4,5,6)P_4, is a potent inhibitor of the Ca^{2+}-dependent Cl^- current. The significance of this phenomenon in epithelial cells may lie in its ability to prevent stimulation of PLC from resulting in a potentially deleterious loss of cellular salt and fluid. It may also be a novel regulatory process that has an impact on the regulation of other physiological activities that rely on careful control of Cl^- efflux. This new knowledge about the signalling effects of Ins(3,4,5,6)P_4 identifies this polyphosphate as a potentially important therapeutic target, because antagonists of Ins(3,4,5,6)P_4 would be expected to increase PLC-mediated, Ca^{2+}-dependent Cl^- permeability in CF patients. This study therefore highlights a new and important area of cell biology with therapeutic significance.

5.2 Future directions

A better understanding of the regulation of Cl^- channels in epithelial cells is necessary to improve CF treatment. It has been demonstrated here that Ins(3,4,5,6)P_4 is an important feedback signalling molecule for the receptor-mediated, Ca^{2+}-dependent Cl^- channel. Emerging evidence has suggested that Ins(3,4,5,6)P_4

does not inhibit the action of calcium/calmodulin-dependent kinase (Xie *et al.*, 1996). It is likely that Ins(3,4,5,6)P_4 might directly interact with the Ca^{2+}-dependent Cl^- channel. It is therefore important to extend the present work to the single channel level to fully understand Ins(3,4,5,6)P_4 action on the Ca^{2+}-dependent Cl^- channel.

Molecular characterization of the Ins(3,4,5,6)P_4 receptor would also be beneficial to CF treatment. By identifying its receptor, an antagonist for Ins(3,4,5,6)P_4 could be developed, and hence, a potentiate CF treatment via increased PLC-mediated, Ca^{2+}-dependent Cl^- conductance. Moreover, the Ins(3,4,5,6)P_4 receptor could be used as a potential marker for isolating the Ca^{2+}-dependent Cl^- channels since inhibition of Ins(3,4,5,6)P_4 is highly specific to this Cl^- conductance. This would provide a major advance in the molecular biology of epithelial Cl^- channels.

BIBLIOGRAPHY

Abraham, E.H., Prat, A.G., Gerweck, L., Seneveratne, T., Arceci, R.J., Kramer, R., Guidotti, G., Cantiello, H.F. (1993) The multidrug resistance (mdr1) gene product functions as an ATP channel. *Proc. Natl. Acad. Sci. USA* 90:312-316.

Ackerman, M.J., Wickman, K.D., and Clapham, D.E. (1994) Hypotonicity activates a native chloride conductance in Xenopus oocytes. *J. Gen. Physiol.* 103:153-179.

Adachi, S., Uchida, S., Ito, H., Hata, M., Hiroe, M., Marumo, F., And Sasaki, S. (1994) Two isoforms of a chloride channel predominantly expressed in thick ascending limb of Henle's loop and collecting ducts of rat kidney. *J. Biol. Chem.* 269:17677-17683.

Adorante, J.S. and Cala, P.M. (1995) Mechanisms of regulatory volume decrease in nonpigmented human ciliary epithelial cells. *Am. J. Physiol.* 268:C721-C731.

Anderson, M.P., Berger, H.A., Rich, D.P., Gregory, R.J., Smith, A.E., and Welsh, M.J. (1991a) Nucleoside trisphosphates are required to open the CFTR chloride channel. *Cell* 67:775-784.

Anderson, M.P., Gregory, R.J., Thompson, S., Souza, D.W., Paul, S., Mulligan, R.C., Smith, A.E., and Welsh, M.J. (1991b) Demonstrated that CFTR is a chloride channel by alteration of its anion selectivity. *Science* 253:202-205.

Anderson, M.P., Sheppard, D.N., Berger, H.A., and Welsh, M.J. (1992) Chloride channels in the apical membrane of normal and cystic fibrosis airway and intestinal epithelia. *Am. J. Physiol.* 263:L1-L14.

Anderson, M.P. and Welsh, M.J. (1991) Calcium and cAMP activated different chloride channels in apical membrane of normal and cystic fibrosis epithelia. *Proc. Natl. Acad. Sci. USA* 88:6003-6007.

Ausiello, D.A., Stow, J.L., Cantiello, H.F., de Almeida, J.B., and Benos, D.J. (1992) Purified epithelial Na^+ channel complex contains the pertussis toxin-sensitive $G\alpha_{i\text{-}3}$ protein. *J. Biol. Chem.* 267:4759-4765.

Balboa, M., Firestein, B.L., Godson, C., Bell, K.S., and Insel, P.A. (1994) Protein kinase $C\alpha$ mediates phospholipase D activation by nucleotides and phorbol ester in Madin-Darby canine kidney cells. Stimulation of phospholipase D is independent of polyphosphoinositide-specific phospholipase C and phospholipase A_2. *J. Biol. Chem.* 269:10511-10516.

Balla, T., Baukal, A.J., and Catt, K.J. (1988) Multiple pathways of inositol polyphosphate metabolism in angiotensin-stimulated adrenal glomerulosa cells. *J. Biol. Chem.* 263:4083-4091.

Balla, T., Sim, S.S., Baukal, A.J., Rhee, S.G., and Catt, K.J. (1994) Inositol polyphosphates are not increase by overexpression of Ins(1,4,5)P_3 3-kinase but show cell-cycle dependent changes in growth factor-stimulated fibroblasts. *Mol. Biol. Cell* 5:17-27.

Balla, T., Sim, S.S., Iida, T., Choi, K.Y., Catt, K.J., and Rhee, S.G. (1991) Agonist-induced calcium signalling is impaired in fibroblasts overproducing inositol 1,3,4,5-tetrakisphosphate. *J. Biol. Chem.* 266:24719-24726.

Banderali, U. and Roy, G. (1992) Activation of K^+ and Cl^- channels in MDCK cells during volume regulation in hypotonic media. *J. Membrane Biol.* 126:219-234.

Bean, B.P. (1992) Pharmacology and electrophysiology of ATP-activated ion channels. *Trends Pharmacol. Sci.* 131:87-90.

Bear, C.E., Li, C., Kartner, N., Bridges, R.J., Jensen, T.J., Ramjeesingh, M., and Riordan, R.J. (1992) Purification and functional reconstitution of cystic fibrosis transmembrane conductance regulator (CFTR). *Cell* 68:809-818.

Bear, C.E. and Reyes, E.F. (1992) cAMP-activated chloride conductance in the colonic cell line, Caco-2. *Am. J. Physiol.* 262:C251-256.

Benos, D.J., Awayda, M.S., Ismailov, I.I., and Johnson, J.P. (1995) Structure and function of amiloride-sensitive Na^+ channels. *J. Membrane Biol.* 143:1-18.

Benos, D.J., Saccomani, G., Brenner, B.M., and Sariban-Sohraby, S. (1987) The epithelial sodium channel. Subunit number and location of the amiloride binding site. *J. Biol. Chem.* 262:10613-10618.

Berkner, K.L. (1988) Development of adenovirus vectors for the expression of heterologous genes. *Biotechniques* 6:616-629.

Berridge, M.J. and Irvine, R.F. (1989) Inositol phosphates and cell signalling. *Nature* 341:197-205.

Berschneider, H.M., Knowles, M.R., Azizkhan, R.G., Boucher, R.C., Tobey, N.A., Orlando, R.C., and Powell, D.W. (1988) Altered intestinal chloride transport in cystic fibrosis. *FASEB J.* 2:2625-2629.

Best, L. Sheader, E.A., and Brown, P.D. (1996) A volume-activated anion conductance in insulin-secreting cells. *Pflügers Arch.* 431:363-370.

Beyer, E.C. and Steinberg, T.H. (1991) Evidence that gap junction protein connexin-43 is the ATP-induced pore of mouse macrophages. *J. Biol. Chem.* 266:7971-7974.

Bezprozvanny, I. and Ehrlich, B.E. (1995) The inositol 1,4,5-trisphosphate (InsP_3) receptor. *J. Membrane Biol.* 145:205-216.

Bird, G. St J., Rossier, M.F., Hughes, A.R., Shears, S.B., Armstrong, D.L., and Putney, J.W. Jr. (1991) Activation of Ca^{2+} entry into acinar cells by a non-phosphorylatable inositol trisphosphate. *Nature* 352:162-165.

Bird, G. St J. and Putney, J.W. Jr. (1996) Effect of inositol 1,3,4,5-tetrakisphosphate on inositol trisphosphate-activated Ca^{2+} signalling in mouse lacrimal acinar cells. *J. Biol. Chem.* 271:6766-6770.

Blanchard, K., Hoffman, S.L., and Djeu, J.Y. (1995) Inhibition of extracellular ATP-mediated lysis of human macrophages by calmodulin antagonists. *J. Cell. Biochem.* 57:452-464.

Boucher, R.C., Cheng, E.-H., Paradiso, A.M., Stutts, M.J., Knowles, M.R., and Earp, H.S. (1989) Chloride secretion response of cystic fibrosis human airway epithelia. Preservation of calcium but not protein kinase C- and A-dependent mechanisms. *J. Clin. Invest.* 84:1424-1431.

Boucher, R.C., Stutts, M.J., Knowles, M.R., Cantley, L., and Gatzy, J.T. (1986) Na^+ transport in cystic fibrosis respiratory epithelium: abnormal basal rate and response to adenylate cyclase activation. *J. Clin. Invest.* 78:1245-1252.

Boyer, J.L., Lazarowski, E.R., Chen, X.-H., and Harden, T.K. (1993) Identification of a P_{2Y}-purinergic receptor that inhibits adenylyl cyclase but does not activate phospholipase C. *J. Pharmacol. Exp. Ther.* 267:1140-1146.

Brake, A.J., Wagenbach, M.J., and Julius, D. (1994) New structural motif for ligand-gated channels defined by an ionotropic ATP receptor. *Nature* 371:519-523.

Brandt, S. and Jentsch, T.J. (1995) ClC-6 and ClC-7 are two novel broadly expressed members of the ClC chloride channel family. *FEBS Lett.* 377:15-20.

Bretschneider, F., Klapperstück, M., Löhn, M., and Markwardt, F. (1995) Nonselective cationic currents elicited by extracellular ATP in human B-lymphocytes. *Pflügers Arch.* 429:691-698.

Brown, D.M. (1968) Aspects of phosphate ester chemistry as applied to phosphoinositides. *Ann. NY Acad. Sci.* 165:687-694.

Burgess, G.M., Bird, G.S., Obie, J.F., and Putney, J.W. Jr. (1991) The mechanism for synergism between phospholipase C- and adenylyl cyclase-linked hormones in liver. Cyclic AMP-dependent kinase augments inositol trisphosphate-mediated Ca^{2+} mobilization without increasing the cellular levels of inositol polyphosphates. *J. Biol. Chem.* 266:4772-4781.

Burnstock, G. (1978) Cell membrane receptors for drugs and hormones: A multidisciplinary approach. Raven Press, New York, pp.107-118.

Canessa, C.M., Horisberger, J.-D., and Rossier, B.C. (1993) Epithelial sodium channel related to proteins involved in neurodegeneration. *Nature* 361:467-470.

Canessa, C.M., Schild, L., Buell, G., Thorens, B., Gautschil, I., Horisberger, J.-D., and Rossier, B.C. (1994) Amiloride-sensitive epithelial sodium channel is made of three homologous subunits. *Nature* 367:463-467.

Cantiello, H.F., Prat, A.G., Reisin, I.L., Ercole, L.B., Abraham, E.H., Amara, J.F., Gregory, R.J., and Ausiello, D.A. (1994) External ATP and its analogs activate the cystic fibrosis transmembrane conductance regulator by a cyclic AMP-independent mechanism. *J. Biol. Chem.* 269:11224-11232.

Cass, C.E. (1995) Nucleoside transport. In Drug transport in antimicrobial and anticancer chemotherapy. Ed., Georgopapadakou, N.H., Marcel, Dekker, New York, pp. 403-451.

Castillo, C.J., Moro, M.A., Del Valle, M., Sillero, A., Garcia, A.G., and Sillero, M.A. (1992) Diadenosine tetraphosphate is co-released with ATP and catecholamines from bovine adrenal medulla. *J. Neurochem.* 59:723-732.

Chan, H.-C., Goldstein, J., and Nelson, D.J. (1992) Alternative pathways for chloride conductance activation in normal and cystic fibrosis epithelial cells. *Am. J. Physiol.* 262:C1273-1283.

Chang, X.-B., Tabcharani, J.A., Hou, Y.-X., Jensen, T.J., Kartner, N., Alon, N., Hanrahan, J.W., Riordan, J.R. (1993) Protein kinase A still activates CFTR chloride channel after mutagenesis of all 10 PKA consensus phosphorylation sites. *J. Biol. Chem.* 268:11304-11311.

Chao, A.C., Zifferblatt, J.B., Wagner, J.A., Dong, Y.J., Gruenert, D.C., and Gardner, P. (1994) Stimulation of chloride secretion by P_1 purinoceptor agonists in cystic fibrosis phenotype airway epithelial cell line CFPEo-. *Br. J. Pharmacol.* 112:169-175.

Chen, Z.P., Kratzmeier, M., Poch, A., Xu, S., McArdle, C.A., Levy, A., Mukhopadhyay, A.K., and Lightman, S.L. (1996) Effects of extracellular nucleotides in the pituitary: adenosine trisphosphate receptor-mediated intracellular responses in gonadotrope-derived alpha T3-1 cells. *Endocrinol.* 137:248-256.

Chern, Y., Lai, H.L., Fong, J.C., and Liang, Y. (1993) Multiple-mechanisms for desensitization of A_{2a} adenosine receptor-mediated cAMP elevation in rat pheochromocytoma PC12 cells. *Mol. Pharmacol.* 44:950-958.

Civan, M.M., Marano, C.W., Matschinsky, F.W., and Peterson-Yantorno, K. (1994) Prolonged incubation with elevated glucose inhibits the regulatory response to shrinkage of cultured human retinal pigment epithelial cells. *J. Membrane Biol.* 139:1-13.

Clarke, L.L. and Boucher, R.C. (1992) Chloride secretion response to extracellular ATP in human normal and cystic fibrosis nasal epithelia. *Am. J. Physiol.* 263:C348-C356.

Clark, L.L., Grubb, B.R., Yankaskas, J.R., Cotton, C.U., McKenzie, A., and Boucher, R.C. (1994) Relationship of a non-cystic fibrosis transmembrane conductance regulator-mediated chloride conductance to organ-level disease in *Cftr*(-/-) mice. *Proc. Natl. Acad. Sci. USA* 91:479-483.

Clemens, M.G. and Forrester, T. (1981) Appearance of adenosine triphosphate in the coronary sinus effluent from isolated working rat heart in response to hypoxia. *J. Physiol.* 312:143-158.

Cliff, W.H. and Frizzell, R.A. (1990) Separate Cl^- conductances activated by cAMP and Ca^{2+} in Cl^--secreting epithelial cells. *Proc. Natl. Acad. Sci. USA.* 87: 4956-4960

Cockcroft, S. and Stutchfield, J. (1989) ATP stimulates secretion in human neutrophils and HL60 cells via a pertussis toxin-sensitive guanine nucleotide-binding protein coupled to phospholipase C. *FEBS Lett.* 245:25-29.

Colman, R.W. (1990) Aggregin: a platelet ADP receptor that mediates activation. *FESEB J.* 4:1425-1435.

Cooley, J.W., Lewis, P.A.W., and Welch, P.D. (1970) The fast fourier transform algorithm: Programming considerations in the calculation of sine, cosine and laplace transforms. *J. Sound Vibrations* 12:315.

Copello, J., Segal, Y., and Reuss, L. (1991) Cytosolic pH regulates maxi K^+ channels in Necturus gall-bladded epithelial cells. *J. Physiol.* 434:577-590.

Coutinho-Silva, R., Alves, L.A., Savino, W., and Persechini, P.M. (1996) A cation non-selective channel induced by extracellular ATP in macrophages and phagocytic cells of the thymic reticulum. *Biochim. Biophys. Acta* 1278:125-130.

Craxton, A., Erneux, C., and Shears, S.B. (1994) Inositol 1,4,5,6-tetrakisphosphate is phosphorylated in rat liver by a 3-kinase that is distinct from inositol 1,4,5-trisphosphate 3 kinase. *J. Biol. Chem.* 269:4337-4342.

Cuppoletti, J., Baker, A.M., and Malinowska, D.H. (1993) Cl^- channel of the gastric parietal cell that are active at low pH. *Am. J. Physiol.* 264:C1609-C1618.

Dalemans, W., Barbry, P., Champigny, G., Jallat, S., Dott, K., Dreyer, D., Crystal, R.G., Pavirani, A., Lecocq, J.P., and Lazdunski, M. (1991) Altered chloride ion channel kinetics associated with the delta F508 cystic fibrosis mutation. *Nature* 354:526-528.

Dalziel, H.H. and Westfall, D.P. (1994) Receptors for adenine nucleotides and nucleosides: Subclassification, distribution, and molecular characterization. *Pharmacol. Rev.* 46:449-466.

Daniel, J.L., Dangelmaier, C.A., Selak., M., and Smith, J.B. (1986) ADP stimulates IP_3 formation in human platelets. *FEBS Lett.* 206:299-303.

DeCoursey, T.E., Jacobs, E.R., and Silver, M.R. (1988) Potassium currents in rat type II alveolar epithelial cells. *J. Physiol.* 395:487-505.

DeLisle, S., Mayr, G.W.,and Welsh, M.J. (1995) Inositol phosphate structural requisites for Ca^{2+} influx. *Am. J. Physiol.* C1485-C1491.

DeLisle, S., Radenberg, T., Wintermantel, M.R., Tietz, C., Parys, J.B., Pittet, D., Welsh, M.J., and Mayr, G.W. (1994) Second messenger specificity of the inositol trisphosphate receptor: reappraisal based upon novel inositol phosphates. *Am. J. Physiol.* 266:C429-C436.

Desir, G.V., Hamline, H.A., Puente, E., Reilly, R.F., Hildebrandt, F., and Igarashi, P. (1992) Isolation of putative voltage-gated epithelial K-channel isoforms from rabbit kidney and LLC-PK_1 cells. *Am. J. Physiol.* 262:F151-F157.

Devor, D.C. and Frizzell, R.A. (1993) Calcium-mediated agonists activate an inwardly rectified K^+ channel in colonic secretory cells. *Am. J. Physiol.* 265:C1271-C1280.

Dharmsathaphorn, K., McRoberts, J.A., Mandel, K.G., Tisdale, L.D., and Masui, H. (1984) A human colonic tumour cell line that maintains vectorial electrolyte transport. *Am. J. Physiol.* 246:G204-G208

Díaz, M., Valverde, M., Higgins, C.F., Rucareanu, C., and Sepúlveda, F.V. (1993) Volume-activated chloride channels in Hela cells are blocked by verapamil and dideoxyforskolin. *Pflügers Arch.* 422:347-353.

Diener, M., Nobles, M., and Rummel, W. (1992) Activation of basolateral Cl^- channels in the rat colonic epithelium during regulatory volume decrease. *Pflügers Arch.* 421:530-538.

Di Virgilio, F. (1995) The P_{2Z} purinoceptor: an intriguing role in immunity, inflammation and cell death. *Immunol. Today* 16:524-528.

Drury, A.N. and Szent-Gyorgyi, A. (1929) The physiological activity of adenine compounds with special reference to their action upon mammalian heart. *J. Physiol.* 68:231-237.

Dubyak, G.R. (1991) Signal transduction by P_2-purinergic receptors for extracellular ATP. *Am. J. Respir. Cell Mol. Biol.* 4:295-300.

Dubyak, G.R. and El-Moatassim, C. (1993) Signal Transduction via P_2-purinergic receptors for extracellular ATP and other nucleotides. *Am. J. Physiol.* 265:C577-C606.

Duszyk, M., French, A.S., and Man, S.F.P. (1992) Noise analysis and single-channel observations of 4 pS chloride channels in human airway epithelia. *Biophys. J.* 61:583-587.

Duszyk, M., French, A.S., Man, S.F.P. and Becker, A.B. (1991) An inwardly rectifying chloride channel in rageed-sensitized canine tracheal epithelial cells. *Eur. Biophys. J.* 20:65-69.

Duszyk, M., French, A.S., and Man, S.F.P. (1989) Cystic fibrosis affects chloride and sodium channels in human airway epithelia. *Can. J. Physiol. Pharmacol.* 67:1362-1365.

Duszyk, M., Liu, D., French, A.S., and Man, S.F.P. (1993) Halide permeation through three types of epithelial anion channels after reconstitution into giant liposomes. *Eur. Biophys. J.* 22:5-11.

Duszyk, M., Liu, D., Kamosinska, B., French, A.S., and Man, S.F.P. (1995) Characterization and regulation of a chloride channel from bovine tracheal epithelium. *J. Physiol.* 489:81-93.

Edelman J.L., Loo, D.D., and Sachs, G. (1995) Characterization of potassium and chloride channels in the basolateral membrane of bovine nonpigmented ciliary epithelial cells. *Invest. Ophtha. Vis. Sci.* 36:2706-2716.

Egan, M., Flotte, T., Afione, S., Solow, R., Zeitlin, P.L., Carter, B.J., and Guggino, W.B. (1992) Defective regulation of outwardly rectifying Cl^- channels by protein kinase A corrected by insertion of CFTR. *Nature* 358:581-584.

Ehrenstein, G., Lecar, H., and Nossal, R. (1970) The nature of the negative resistance in biomolecular lipid membranes containing excitability-inducing material. *J. Gen. Physiol.* 55:119-133

Eveloff, J.L. and Warnock, D.G. (1987) Activation of ion transport systems during cell volume regulation. *Am. J. Physiol.* 252:F1-F10.

Feoktistov, I. and Biaggioni, I. (1995) Adenosine A_{2b} receptors evoke interleukin-8 secretion in human mast cells. An enprofylline-sensitive mechanism with implications for asthma. *J. Clin. Invest.* 96:1979-1986.

Ferris, C.D., Huganir, R.L., Bredt, D.S., Cameron, A.M., and Synder, S.H. (1991) Inositol trisphosphate receptor: phosphorylation by protein kinase C and calcium calmodulin-dependent protein kinases in reconstituted lipid vesicles. *Proc. Natl. Acad. Sci. USA* 88:2232-2235.

Ferris, C.D., Huganir, R.L., and Synder, S.H. (1990) Calcium flux mediated by purified inositol 1,4,5-trisphosphate receptor in reconstituted lipid vesicles is allosterically regulated by adenine nucleotides. *Proc. Natl. Acad. Sci. USA* 87:2147-2151.

Fieber, L. and Adams, D.J. (1991) Adenosine triphosphate-evoked currents in cultured neurons dissociated from rat parasympathetic cardiac ganglia. *J. Physiol.* 434:239-256.

Forrester, T. (1990) Release of ATP from heart: Presentation of a release model using human erythrocyte. *Ann. NY Acad. Sci.* 603:335-352.

Freshney, R.I. (1992) In Culture of specialized cells. Culture of epithelial cells. Ed., Fresnney, R.I., Wiley-Liss, Inc., New York, New York, pp. 1-24.

Frizzell, R.A. and Halm, D.R. (1990) Chloride channels in epithelial cells. In Current topics in membranes and transport, Vol 37.: Channels and noise in epithelial tissues. Eds., Helman, S.I. and Van Driessche, W., Academic Press, San Diego, California, pp. 247-282.

Frizzell, R.A., Rechkemmer, G., and Shoemaker, R.L. (1986) Altered regulation of airway epithelial cell chloride channels in cystic fibrosis. *Science* 233:558-560.

Fuller, C.M., Ismailov, I.I., Keeton, D.A., Benos, D.J. (1994) Phosphorylation and activation of a bovine tracheal anion channel by Ca^{2+}/calmodulin-dependent protein kinase II. *J. Biol. Chem.* 269:26642-26650.

Furlong, T.J., Pierce, K.D., Selbie, L.A., and Shine, J. (1992) Molecular characterization of a human brain adenosine A_2 receptor. *Mol. Brain Res.* 15:62-66.

Gabriel, S.E., Clarke, L.L., Boucher, R.C., and Stutts, M.J. (1993) CFTR and outward rectifying chloride channels are distinct proteins with regulatory relationship. *Nature* 363:263-266.

Galietta, L.J.V., Zegarra-Moran, O., Mastrocola, T., Wöhrle, C., Rugolo, M., and Romeo, G. (1994) Activation of Ca^{2+}-dependent K^+ and Cl^- currents by UTP and ATP in CFPAC-1 cells. *Pflügers Arch.* 426:534-541.

Gerber, U. and Gahwiler, B.H. (1994) $GABA_B$ and adenosine receptors mediate enhancement of the K^+ current, I_{AHP}, by reducing adenylyl cyclase activity in rat CA3 hippocampal neurons. *J. Neurophysiol.* 72:2360-2367.

Gerwins, P. and Fredholm, B.B. (1992) Stimulation of adenosine A_1 receptors and bradykinin receptors, which act via different G proteins, synergistically raises inositol 1,4,5-trisphosphate and intracellular free calcium in DDT1MF-2 smooth muscle cells. *Proc. Natl. Acad. Sci. USA* 89:7330-7334.

Gibson, T.G., Hyvonen, M., Musacchio, A., Saraste, M., and Birney, E. (1994) PH domian: the first anniversary. *Trends Biochem. Sci.* 19:349-353.

Gill, D.R., Hyde, S.C., and Higgins, C.F. (1992) Volume-regulated chloride channels associated with the human multidrug-resistance P-glycoprotein. *Nature* 355:830-833.

Gottesman, M.M. and Pastan, I. (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. *Ann. Rev. Biochem.* 62:385-427.

Grinstein, S., Clarke, C.A., Rothstein, A., and Gelfand, E.W. (1983) Volume-induced anion conductance in human B lymphocytes is cation independent. *Am. J. Physiol.* 245:C160-C163.

Gründer, S., Thiemann, A., Pusch, M., and Jentsch, M. (1992) Regions involved in the opening of ClC-2 chloride channel by voltage and cell volume. *Nature* 360:759-762.

Grygorczyk, R., Tabcharani, J.A., and Hanrahan, J.W. (1996) CFTR channels expressed in CHO cells do not have detectable ATP conductance. *J. Membrane Biol.* 151:139-148.

Gschwentner, M., Nagl, U.O., Wöll, E., Schmarda, A., Ritter, M., and Paulmichl, M. (1995) Antisense oligonucleotides suppress cell-volume-induced activation of chloride channels. *Pflügers Arch.* 430:464-470.

Hajjar, J.J., Aziz, W., Molski, T.F., and Sháafi, R.I. (1995) Stimulation of intestinal Na^+/H^+ exchange by cell volume changes during fasting and refeeding in rats. *Proc. Soc. Exp. Biol. Med.* 209:354-359.

Handler, J.S. and Kwon, H.M. (1993) Regulation of renal cell organic osmolyte transport by tonicity. *Am. J. Physiol.* 265:C1449-1455.

Hanrahan, J.W., Alles, W.P., and Lewis, S.A. (1985) Single anion-selective channels in basolateral membrane of a mammalian tight epithelium. *Proc. Natl. Acad. Sci. USA* 82:7791-7795.

Hawkins, P.T., Poyner, D.R., Jackson, T.R., Letcher, A.J., Lander, D.A., and Irvine, R.F. (1993) Inhibition of iron-catalysed hydroxyl radical formation by inositol polyphosphates: A possible physiological function for *myo*-inositol hexakisphosphate. *Biochem. J.* 294:929-934.

Haws, C.M., Krouse, M.E., Xia, Y., Gruenert, D.C., and Wine, J.J. (1992) CFTR channels in immortalized human airway cells. *Am. J. Physiol.* 263:L692-707.

Hille, B. (1992) Ionic Channels of Excitable Membranes. Sinauer, Sunderland, Massachusetts, pp. 13 & 317.

Ho, K., Nichos, C.G., Lederer, W.J., Lytton, J., Vassilev, P.M., Kanazirska, M.V., and Hebert, S.C. (1993) Cloning and expression of an inwardly rectifying ATP-regulated potassium channel. *Nature* 362:31-37.

Hodson, M.E. and Shah, P.L. (1995) DNase trials in cystic fibrosis. *Eur. Respir. J.* 8:1786-1791.

Hoth, M. and Penner, R. (1992) Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature* 355:353-356.

Hughes, P.J., Hughes, A.R., Putney, J.W. Jr., and Shears, S.B. (1989) The regulation of the phosphorylation of inositol 1,3,4-trisphosphate in cell-free preparations and its relevance to the formation of inositol 1,3,4,6-tetrakisphosphate in agonist-stimulated rat parotid acinar cells. *J. Biol. Chem.* 264:19871-19878.

Hughes, P.J. and Shears, S.B. (1990) Inositol 1,3,4,5,6-pentakisphosphate and inositol hexakisphosphate inhibit Inositol 1,3,4,5-tetrakisphosphate 3 phosphatase in rat parotid glands. *J. Biol. Chem.* 265:9869-9875.

Hwang, T.-C., Guggino, S.E., and Guggino, W.B. (1990) Direct modulation of secretory chloride channels by arachidonic and other *cis*-unsaturated fatty acids. *Proc. Natl. Acad. Sci. USA* 87:5706-5709.

Iino, M. (1990) Biphasic Ca^{2+} dependence of inositol 1,4,5-trisphosphate-induced Ca release in smooth muscle cells of the guinea pig taenia caeci. *J. Gen. Physiol.* 95:1103-1122.

Ikeda, K., Suzuki, M., Furukawa, M., and Takasaka, T. (1995) Calcium mobilization and entry induced by extracellular ATP in the non-secretory epithelial cell of the cochlear lateral wall. *Cell Calcium* 18:89-99.

Irvine, R.F. (1992) Inositol phosphates and Ca^{2+} entry: toward a proliferation or a simplification? *FASEB J.* 6:3085-3091.

Irvine, R.F., Änggård, E.E., Letcher, A.J., and Downes, C.P. (1985) Metabolism of inositol 1,4,5-trisphosphate and inositol 1,3,4-trisphosphate in rat parotid acinar cells. *Biochem. J.* 229:505-511.

Ivorra, I., Gigg, R., Irvine, R.F., and Parker, I. (1991) Inositol 1,3,4,6-tetrakisphosphate mobilizes calcium in Xenopus oocyte with high potency. *Biochem. J.* 273:317-321.

Jan, L.Y. and Jan, Y.N. (1992) Tracing the roots of ion channels. *Cell* 69:715-718.

Jentsch, T.J. (1996) Chloride channels: a molecular perspective. *Cur. Opin. Neurobiol.* 6:303-310.

Jentsch, T.J., Günther, W., Pusch, M., and Schwappach, B. (1995) Properties of voltage-gated chloride channels of the ClC gene family. *J. Physiol.* 482:19S-25S.

Jentsch, T.J., Steinmeyer, K., and Schwarz, G. (1990) Primary structure of *Torpedo marmorata* chloride channel isolated by expression cloning in *Xenopus* oocytes. *Nature* 348:510-514.

Ji, H., Sandberg, K., Baukal, A.J., and Catt, K.J. (1989) Metabolism of inositol pentakisphosphate to inositol hexakisphosphate in Xenopus laevis oocytes. *J. Biol. Chem.* 264:20185-20188.

Jiang, C., Finkbeiner, W.E., Widdicombe, J.H., McCray, P.B., and Miller, S.S. (1993) Altered fluid transport across airway epithelium in cystic fibrosis. *Science* 262:424-427.

Kachintorn, U., Vajanaphanich, M., Barrett, K.E., and Traynor-Kaplan, A.E. (1993) Elevation of inositol tetrakisphosphate parallels inhibition of Ca^{2+}-dependent Cl^- secretion in T84 cells. *Am. J. Physiol.* 264:C671-C676.

Kaczorowski, G.J., Knaus, H.-G., Leonard, R.J., McManus, O.B., and Garcia, M.L. (1996) High-conductance calcium-activated potassium channels; structure, pharmacology, and function. *J. Bioenergetics Biomem.* 28:255-267.

Kalinoski, D.L., Aldinger, S.B., Boyle, A.G., Hugue, T., Marecek, J.F., Prestwich, G.D., and Restrepo, D. (1992) Characterization of a novel inositol 1,4,5-trisphosphate receptor in isolated olfactory cilia. *Biochem. J.* 281:449-456.

Kanematsu, T., Misumi, Y., Watanabe, Y., Ozaki, S., Koga, T., Iwanaga, S., Ikehara, Y., and Hirata, M. (1996) New Ins(1,4,5)P_3-binding protein similar to phospholipase Cδ-1. *Biochem. J.* 313:319-325.

Kanwar, R.K., Ganguly, N.K., Kumar, L., Rakesh, J., Panigrahi, D., and Walia, B.N. (1995) Calcium and protein kinase C play an important role in Campylobacter jejuni-induced changes in Na^+ and Cl^- transport in rat ilem in vitro. *Biochim. Biophys. Acta.* 1270:179-192.

Kawasaki, M., Uchida, S., Monkawa, T., Miyawaki, A., Mikoshiba, K., Marumo, F., Sasaki, S. (1994) Cloning and expression of a protein kinase C-regulated chloride channel abundantly expressed in rat brain neuronal cells. *Neuron* 12:597-604.

Kelly, M.E.M., Dixon, S.J., and Sims, S.M. (1994) Outwardly rectifying chloride current in rabbit osteoclasts is activated by hyposmotic stimulation. *J. Physiol.* 475:377-389.

Kennedy, B.G. (1994) Volume regulation in cultured cells derived from human retinal pigment epithelium. *Am. J. Physiol.* 266:C676-683.

Kerem, B., Rommens, J.M., Buchanan, J.A., Markiewicz, D., Cox, T.K., Chakravarti, A., Buchwald, M., and Tsui, L.-C. (1989) Identification of the cystic fibrosis gene: genetic analysis. *Science* 245:1073-1080.

Kesselring, F., Spicher, K., and Porzig, H. (1994) Changes in G protein pattern and in G protein-dependent signalling during erythropoietin- and dimethysulfoxide-induced differentiation of murine erythroleukemia cells. *Blood* 84:4088-4098.

Khan, A.A., Steiner, J.P., Klein, M.G., Schneider, M.F., and Snyder, S.H. (1992) IP_3 receptor: localization to plasma membrane of T cells and cocapping with the T cell receptor. *Science* 257:815-818.

Klär, B., Leipziger, L., Nitschke, R., Greger, R. (1993) Ca^{2+} as a second messenger in CFPAC-1 cells. *Cell Physiol. Biochem.* 3:17-27.

Knowles, M.R., Church, N.L., Waltner, W.E., Yankaskas, J.R., Gilligan, P.H., King, M., Edwards, L.J., Helms, R.W., and Boucher, R.C. (1990) A pilot study of aerosolized amiloride for the treatment of cystic fibrosis lung disease. *N. Engl. J. Med.* 332:1189-1194.

Knowles, M.R., Olivier, K.N., Hohneker, K.H., Robinson, J., Bennett, W.D., and Boucher, R.C. (1995) Pharmacologic treatment of abnormal ion transport in the airway epithelium in cystic fibrosis. *Chest* (2 suppl):71S-76S.

Kohno, Y., Sei, Y., Koshiba, M., Kim, H.O., and Jacobson, K.A. (1996) Induction of apotosis in HL-60 human promyelocytic leukemia cells by adenosine A_3 receptor agonists. *Biochem. Biophys. Res. Com.* 219:904-910.

Komwatana, P., Dinudom, A., Young, J.A., and Cook, D.I. (1994) Characterization of the Cl^- conductance in the granular duct cells of mouse mandibular glands. *Pflügers Arch.* 428:641-647.

Krapivinsky, G.B., Ackerman, M.J., Gordon, E.A., Krapivinsky, L.D., and Clapham, D.E. (1994) Molecular characterization of a swelling-induced chloride conductance regulatory protein, pI_{Cln}. *Cell* 76:439-448.

Kubo, Y., Baldwin, T.J., Jan, Y.N., and Jan, L.Y. (1993) Primary structure and functional expression of a mouse inward rectifier potassium channel. *Nature* 362:127-133.

Kubo, M. and Okada, Y. (1992) Volume-regulatory Cl$^-$ channel currents in cultured human epithelial cells. *J. Physiol.* 456: 351-371.

Kunzelmann, K., Kubitz, R., Grolik, M., Warth, R., and Greger, R. (1992) Small-conductance Cl$^-$ channels in HT29 cells: activation by Ca^{2+}, hypotonic cell swelling and 8-Br-cGMP. *Pflügers Arch.* 421:238-246.

Kunzelmann, K., Pavenstädt, H., Beck, C., Ünal, Ö., Emmrich, P., Arndt, H.J., and Greger, R. (1989a) Characterization of potassium channels in respiratory cells. I. General properties. *Pflügers Arch.* 414:291-296.

Kunzelmann, K., Pavenstädt, H., and Greger, R. (1989) Properties and regulation of chloride channels in cystic fibrosis and normal airway cells. *Pflügers Arch.* 415:172-182.

Lazarowski, E.R., Boucher, R.C., and Harden, T.K. (1994) Calcium-dependent release of archidonic acid in response to purinergic receptor activation in airway epithelium. *Am. J. Physiol.* C406-C415.

Lazarowski, E.R., Mason, S.J., Clarke, L., Harden, T.K., and Boucher, R.C. (1992) Adenosine receptors on human airway epithelia and their relationship to chloride secretion. *Br. J. Pharmacol.* 106:774-782.

Lewis, R.S., Ross, P.E., and Cahalan, M.D. (1993) Chloride channels activated by osmotic stress in T lymphocytes. *J. Gen. Physiol.* 101:801-826.

Li, C., Ramjeesingh, M., and Bear, C.E. (1996) Purified cystic fibrosis transmembrane conductance regulator (CFTR) does not function as an ATP channel. *J. Biol. Chem.* 271:11623-11626.

Li, M., McCann, J.D., Anderson, M.P., Clancy, J.P., Leidtke, C.M., Nairn, A.C., Greengard, P., and Welsh, M.J. (1989) Regulation of chloride channels by protein kinase C in normal and cystic fibrosis airway epithelia. *Science* 244:1353-1356.

Liang, B.T. and Haltiwanger, B. (1995) Adenosine A$_{2a}$ and A$_{2b}$ receptors in cultured fetal chick heart cells. High- and low-affinity coupling to stimulation of myocyte contractility and cAMP accumulation. *Cir. Res.* 76:242-251.

Lin, M., Nairn, A.C., and Guggino, S.E. (1992) cGMP-dependent protein kinase regulation of a chloride channel in T84 cells. *Am. J. Physiol.* 262:C1304-C1312.

Lin, W.-W. and Chuang, D.-M. (1993) Endothelin- and ATP-induced inhibition of adenylyl cyclase activity in C6 glioma cells: role of G$_i$ and calcium. *Mol. Pharmacol.* 44:158-165.

Lindemann, B. and Van Driessche, W. (1977) Sodium-specific membrane channels of frog skin are pores: current fluctuations reveal high turnover. *Science* 195:292-294.

Linden, J. (1994) Cloned adenosine A_3 receptors: Pharmacological properties, species differences and receptor function. *Trends Pharmacol. Sci.* 15:298-306.

Lingueglia, E., Voilley, N., Waldmann, R., Lazdunski, M., and Barbry, P. (1993) Expression cloning of an epithelial amiloride-sensitive Na^+ channel-a new channel type with homologies to *Caenorhabditis elegans* degenerines. *FEBS Lett.* 318:95-99.

Lu, P.-J., Shieh, W.-R., and Chen, C.-S. (1996) Antagonistic effect of inositol pentakisphosphate on inositol trisphosphate receptors. *Biochem. Biophy. Res. Com.* 220:637-642.

Lückhoff, A. and Clapham, D.E. (1992) Inositol 1,3,4,5-tetrakisphosphate activates an endothelial Ca^{2+}-permeable channel. *Nature* 355:356-358.

Lustig, K.D., Shiau, A.K., Brake, A.J., and Julius, D. (1993) Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc. Natl. Acad. Sci. USA* 90:5113-5117.

Ma, H. and Green, R.D. (1992) Modulation of cardiac cyclic AMP metabolism by adenosine receptor agonists and antagonists. *Mol. Pharmacol.* 42:831-837.

MacLeod, R.J. and Hamilton, J.R. (1990) Regulatory volume increase in isolated mammalian jejunal villus is due to bumetanide sensitive NaKCl cotransport. *Am. J. Physiol.* 258:G665-G674.

Maeda, N., Kawasaki, T., Nakade, S., Yokota, N., Taguchi, T., Kasai, M., and Mikoshiba, K. (1991) Structural and functional characterization of inositol 1,4,5-trisphosphate receptor channel from mouse cerebellum. *J. Biol. Chem.* 266:1109-1116.

Mahaut-Smith, M.P., Sage, S.O., and Rink, T.J. (1990) Receptor-activated single channels in intact human platelets. *J. Biol. Chem.* 265:10479-10483.

Malinowska, D.H., Kupert, E.Y., Bahinski, A., Sherry, A.M., and Cuppoletti, J. (1995) Cloning, functional expression, and characterization of a PKA-activated gastric Cl^- channel. *Am. J. Physiol.* 268:C191-C200.

Marunaka, Y. and Eaton, D.C. (1990) Effects of insulin and phosphatase on a Ca^{2+}-dependent Cl^- channel in a distal nephron cell line (A6). *J. Gen. Physiol.* 95:773-789.

Mason, S.J., Paradiso, A.M., and Boucher, R.C. (1991) Regulation of transepithelial ion transport and intracellular calcium by extracellular ATP in human normal and cystic fibrosis airway epithelium. *Br. J. Pharmacol.* 103:1649-1656.

McCann, J.D., Li, M., and Welsh, M.J. (1989) Identification and regulation of whole-cell chloride currents in airway epithelium. *J. Gen. Physiol.* 94:1015-1036.

McEwan G.T.A., Hunter, J., Hirst, B.H., and Simmons, N.L. (1992) Volume-activated Cl^- secrestion and transepithelial vinblastine secretion mediated by P-glycoprotein are not correlated in cultured human T84 intestinal epithelial layers. *FEBS Lett.* 304:233-236.

Meldolesi, J., Madeddu, L., and Pozzan, T. (1990) Intracellular Ca^{2+} storage organelles in non-muscle cells: heterogeneity and functional assignment. *Biochim. Biophy. Acta* 1055:130-140.

Menniti, F.S., Oliver, K.G., Nogimori, K., Obie, J.F., Shears, S.B., and Putney, J.W. Jr. (1990) Oringins of *myo*-inositol tetrakisphosphates in agonist-stimulated rat pancreatoma cells. Stimulation by bombesin of *myo*-inositol 1,3,4,5,6-pentakisphosphate breakdown to *myo*-inositol 3,4,5,6-tetrakisphosphate. *J. Biol. Chem.* 265:11167-11176.

Meyers, K.M., Holmsen, H., and Seachord, C.L. (1982) Comparative study of platelet dense granule constituents. *Am. J. Physiol.* 243:R454-461.

Mignery, G.A., Johnston, P.A., and Sudhof, T.C. (1992) Mechanism of Ca^{2+} inhibition of inositol 1,4,5-trisphosphate (InsP_3) binding to the cerebellar InsP_3 receptor. *J. Biol. Chem.* 267:7450-7455.

Mignery, G.A., Sudhof, T.C., Takei, K., and De Camilli, P. (1989) Putative receptor for inositol 1,4,5-trisphosphate similar to ryanodine receptor. *Nature* 342:192-195.

Mills, D.C, Figures, W.R., Scearce, L.M., Stewart, G.J., Colman, R.F., and Colman, R.W. (1985) Two mechanisms for inhibition of ADP-induced platelet shape change by 5'-p-fluorosulfonylbenzoyladenosine. Conversion to adenosine, and covalent modification at an ADP binding site distinct from that which inhibits adenylyl cyclase. *J. Biol. Chem.* 260:8078-8083.

Morris, A.P. and Frizzell, R.A. (1993) Ca^{2+}-dependent Cl^- channels in undifferentiated human colonic cells (HT-29). I. Single-channel properties. *Am. J. Physiol.* 264:C968-C976.

Morris, A.P., Gallacher, D.V., Irvine, R.F., Petersen, O.H. (1987) Synergism of inositol trisphosphate and tetrakisphosphate in activating Ca^{2+}-dependent K^+ channels. *Nature* 330:653-655.

Morris, A.P., Kirk, K.L., and Frizzell, R.A. (1990) Simultaneous analysis of cell Ca^{2+} and Ca^{2+}-stimulated chloride conductance in colonic epithelial cells (HT-29). *Cell Regulation*. 1:951-963.

Murgo, A.J., Contrera, J.G., and Sistare, F.D. (1994) Evidence for separate calcium-signalling P_{2T} and P_{2U} purinoceptors in human megakaryocytiv Dami cells. *Blood* 83:1258-1267.

Naumov, A.P., Kaznacheyeva, E.V., Kiselyov, K.I., Kuryshev, Y.A., Mamin, A.G., and Mozhayeva, G.N. (1995) ATP-activated inward current and calcium-permeable channel in rat macrophage plasma membranes. *J. Physiol.* 486:323-337.

Neher, E. and Sakmann, B. (1976) Single channel currents recorded from membrane of denervated frog muscle fibres. *Nature* 260:799-802.

Nelson, D.J., Tang, J.M., and Palmer, L.G. (1984) Single-channel recordings of apical membrane chloride conductance in A6 epithelial cells. *J. Membrane. Biol.* 80:81-89.

Nilius, B., Sehrer, J., De Smet, P., Van Driessche, W., and Droogmans, G. (1995a) Volume regulation in a toad epithelial cell line: role of coactivation of K^+ and Cl^- channels. *J. Physio.* 487:367-378.

Nilius, B., Sehrer, J., Heinke, S., and Droogmans, G. (1995b) Ca^{2+} release and activation of K^+ and Cl^- currents by extracellular ATP in distal nephron epithelial cells. *Am. J. Physiol.* 269:C376-C384.

Nilius, B., Sehrer, J., Viana, F., De Dreef, C., Raeymaekers, L., Eggermont, J., Droogmans, G. (1994) Volume-activated Cl^- currents in different mammalian non-excitable cell types. *Pflügers Arch.* 428:364-371.

Nishizuka, Y. (1995) Protein kinase C and lipid signaling for sustained cellular response. *FASEB J.* 9:484-496.

Nogimori, K., Hughes, P.J., Glennon, M.C., Hodgson, M.E., Putney, J.W. Jr., and Shears, S.B. (1991) Purification of an inositol 1,3,4,5-tetrakisphosphate 3-phosphatase from rat liver and the evaluation of its substrate specificity. *J. Biol. Chem.* 266:16499-16506.

Oh, Y. and Benos, D.J. (1993) Single-channel characteristics of a purified bovine renal amiloride-sensitive Na^+ channel in planar lipid bilayers. *Am. J. Physiol.* 264:C1489-C1499.

Oh, Y., Smith, P.R., Bradford, A.L., Keeton, D., and Benos, D.J. (1993) Regulation by phosphorylation of purified epithelial Na^+ channels in planar lipid bilayers. *Am. J. Physiol.* 265:C85-C91.

Ohlmann, P., Laugwitz, K.L., Nurnberg, B., Spicher, K., Schultz, G., Cazenave, J.P., and Gachet, C. (1995) The human platelet ADP receptor activates G_{i2} proteins. *Biochem. J.* 312:775-779.

Olah, M.E. and Stiles, G.L. (1995) Adenosine receptor subtypes: Characterization and therapeutic regulation. *Annu. Rev. Pharmacol. Toxicol.* 35:581-606.

Oliver, K.G., Putney, J.W. Jr., Obie, J.F., and Shears, S.B. (1992) The interconversion of inositol 1,3,4,5,6-pentakisphosphate and inositol tetrakisphosphate in AR4-2J cells. *J. Biol. Chem.* 267:21528-21534.

Olsson, R.A. and Pearson, J.D. (1990) Cardiovascular purinoceptors. *Physiol. Rev.* 70:761-845.

Osipchuk, Y. and Cahalan, M. (1992) Cell-to-cell spread of calcium signals mediated by ATP receptors in mast cells. *Nature Lond.* 359:241-244.

Palczewski, K., Rispoli, G., and Detwiler, P.B. (1992) The influence of arrestin (48K protein) and rhodopsin linase on visual transduction. *Neuron* 8:117-126.

Palmer, L.G. (1992) Epithelial Na channels: function and diversity. *Annu. Rev. Physiol.* 54:51-66.

Parekh, A.B., Terlau, H., and Stühmer, W. (1993) Depletion of InsP_3 stores activates a Ca^{2+} and K^+ current by means of a phosphatase and a diffusible messenger. *Nature* 364:814-818.

Parent, L., Cardinal, J., and Sauvé, R. (1988) Single-channel analysis of a K channel at basolateral membrane of rabbit proximal convoluted tubule. *Am. J. Physiol.* 254:F105-F113.

Park, K.P., Beck, J.S., Douglas, I.J., Brown, P.D. (1994) Ca^{2+}-activated K^+ channels are involved in regulatory volume decrease in acinar cells isolated from the rat lacrimal gland. *J. Membrane Biol.* 141:193-201.

Parker, I. and Ivorra, I. (1991) Inositol tetrakisphosphate liberates stored Ca^{2+} in Xenopus oocytes and facilitates responses to inositol trisphosphate. *J. Physiol.* 433:207-227.

Parr, C.E., Sullivan, D.M., Paradiso, A.M., Lazarowski, E.R., Burch, L.H., Olsen, J.C., Erb, L., Weisman, G.A., Boucher, R.C., and Turner, J.T. (1994) Cloning and expression of a human P_{2U} nucleotide receptor, a target for cystic fibrosis pharmacotherapy. *Proc. Natl. Acad. Sci. USA* 91:3275-3279.

Paulmichl, M., Li, Y., Wickman, K., Ackerman, M., Peralta, E., and Clapham, D. (1992) New mammalian chloride channel identified by expression cloning. *Nature* 356:238-241.

Pavenstädt, H., Spath, M., Schlunck, G., Nauck, M., Fischer, R., Wanner, C., and Schollmeyer, P. (1992) Effect of nucleotides on the cytosolic free calcium activity and inositol phosphate formation in human glomerular epithelial cells. *Br. J. Pharmacol.* 107:189-195.

Pfeilschifter, J. and Merriweather, C. (1993) Extracellular ATP and UTP activation of phospholipase D is mediated by protein kinease C_{ϵ} in rat renal mesangial cells. *Br. J. Pharmacol.* 110:847-853.

Picot, R., Das, I., and Reid, L. (1978) Pus, deoxyribonucleic acid and sputum viscosity. *Thorax.* 33:235-242.

Post., S.R., Jacobson, J.P., and Insel, P.A. (1996) P_2 purinergic receptor agonists enhance cAMP production in Madin-Darby canine kidney epithelial cells via an autocrine/paracrine mechanism. *J. Biol. Chem.* 271:2029-2032.

Prat, A.G., Bertorello, A.M., Ausiello, D.A., and Cantiello, H.F. (1993) Activation of epithelial Na^+ channels by protein kinase-A requires actine filaments. *Am. J. Physiol.* 265:C224-C233.

Press, W.H., Flannery, B.P., Teukolsky, S.A., and Vetterling, W.T. (1990) Numerical Recipes in C. Cambridge University Press, Cambridge, UK.

Putney, J.W. Jr. (1986) A model for receptor-regulated calcium entry. *Cell Cal.* 7:1-12.

Quinton, P.M. (1990) Cystic fibrosis: a disease in electrolyte transport. *FASEB J.* 4:2709-2717.

Quinton, P.M. (1983) Chloride impermeability in cystic fibrosis. *Nature* 301:421-422.

Quinton, P.M. and Reddy, M.M. (1992) Control of CFTR chloride conductance by ATP levels through non-hydrolytic binding. *Nature* 360:79-81.

Randriamampita, C. and Tsien, R.Y. (1993) Emptying of intracellular Ca^{2+} store releases a novel small messenger that stimulates Ca^{2+} influx. *Nature* 364:809-814.

Rasola, A., Gelietta, L.J.V., Gruenert, D.C., and Romeo, G. (1994) Volume-sensitive chloride currents in four epithelial cell lines are not directly correlated to the expression of the MDR-1 gene. *J. Biol. Chem.* 269:1432-1436.

Reddy, M.M., Quinton, P.M., Haws, C., Wine, J.J., Grygorczyk, R., Tabcharani, J.A., Hanrahan, J.W., Gunderson, K.L., and Kopito, R.R. (1996) Failure of the cystic transmembrane conductance regulator to conduct ATP. *Science* 271:1876-1879.

Reisin, I.L., Prat, A.G., Abraham, E.H., Amara, J.F., Gregory, R.J., Ausiello, D.A., and Cantiello, H.F. (1994) The cystic fibrosis transmembrane conductance regulator is a dual ATP and chloride channel. *J. Biol. Chem.* 269:20584-20591.

Ren, H. and Stiles, G.L. (1994) Characterization of the human A_1 adenosine receptor gene: evidence for alternative splicing. *J. Biol. Chem.* 269:3104-3110.

Reuss, L. (1983) Basolateral KCl cotransport in NaCl-absorbing epithelium. *Nature* 305:723-726.

Ridge, F.P.G., Duszyk, M., French, A.S. (1996) A large conductance, Ca^{2+}-activated K^+ channel in a human lung epithelial cell line (A549). (Submitted).

Riordan, J.R., Rommens, J.M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.-L., Drumm, M.L., Iannuzzi, M.C., Collins, F.S., and Tsui, L.-C. (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* 245:1066-1073.

Roch, B., Baró, Hongre, A.-S., and Escande, D. (1995) ATP-sensitive K^+ channels regulated by intracellular Ca^{2+} and phosphorylation in normal (T84) and cystic fibrosis (CFPAC-1) epithelial cells. *Pflügers Arch.* 426:355-363.

Rommens, J.M., Iannuzzi, M.C., Kerem, B., Drumm, M.L., Melmer, G., Dean, M., Rozmahel, R., Cole, J.L., Kennedy, D., Hidaka, N., Zsiga, M., Buchwald, M., Riordan, J.R., Tsui, L.-C., and Collins, F.S. (1989) Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science* 1059-1065.

Sage, S.O., Reast, R., and Rink, T.J. (1990) ADP evokes biphasic Ca^{2+} influx in fura-2-loaded human platelets. Evidence for Ca^{2+} entry regulated by the intracellular Ca^{2+} store. *Biochem. J.* 265:675-680.

Sakamoto, H., Kawasaki, M., Uchida, S., Sasaki, S., and Marumo, F. (1996) Identification of a new outwardly rectifying Cl^- channel that belongs to a subfamily of the ClC Cl^- channels. *J. Biol. Chem.* 271:10210-10216.

Sariban-Sohraby, S., Abramow, M., and Fisher, R.S. (1992) Single-channel behavior of a purified epithelial Na^+ channel subunit that binds amiloride. *Am. J. Physiol.* 263:C1111-C1117.

Sariban-Sohraby, S., Burg, M., Wiesmann, W.P., Chiang, P.K., and Johnson, J.P. (1984) Methylation increases sodium transport into A6 apical membrane vesicles: Possible mode of aldosterone action. *Science* 225:745-746.

Sarkadi, B. and Parker, J.C. (1991) Activation of ion transport pathways by changes in cell volume. *Biochem. Biophys. Acta* 1071:407-427.

Sasakawa, N., Sharif, M., and Hanley, M.R. (1995) Metabolism and biological activities of inositol pentakisphosphate and inositol hexakisphosphate. *Biochem. Pharmacol.* 50:137-146.

Sawada, M., Ichinose, M., and Maeno, T. (1989a) Intracellularly injected inositol 1,3,4,5,6-pentakisphosphate induces a slow inward current in identified neurons of *Aplysia Kurodai*. *Brain Res.* 503:167-169.

Sawada, M., Ichinose, M., and Maeno, T. (1989b) Intracellularly injected inositol hexakisphosphate induces a biphasic current in identified neurons of *Aplysia Kurodai*. *Neurosci. Lett.* 106:328-333.

Schoumacher, R.A., Ram, J., Iannuzzi, M.C., Bradbury, N.A., Wallace, R.W., Hon, C.T., Kelly, D.R., Schmid, S.M., Gelder, F.B., and Rado, T.A. (1990) A cystic fibrosis pancreatic adenocarcinoma cell line. *Proc. Natl. Acad. Sci. USA* 87:4012-4016.

Schoumacher, R.A., Shoemaker, R.L., Halm, D.R., Tallant, E.A., Wallace, R.W., and Frizzell, R.A. (1987) Phosphorylation fails to activated chloride channels from cystic fibrosis airway cells. *Nature* 330:752-754.

Schwiebert, E.M., Egan, M.E., Hwang, T.-H., Fulmer, S.B., Allen, S.S., Cutting, G.R., and Guggino, W.B. (1995) CFTR regulates outwardly rectifying chloride channels through an autocrine mechanism involving ATP. *Cell* 81:1063-1073.

Schwiebert, E.M., Light, D.B., Fejes-Toth, G., Naray-Fejes-Toth, A., and Stanton, B.A. (1990) A GTP-binding protein activates chloride channels in a renal epithelium. *J. Biol. Chem.* 265:7725-7728.

Schwiebert, E.M., Mills, J.W., and Stanton, B.A. (1994) Actin-based cytoskeleton regulates a chloride channel and cell volume in a renal cortical collecting duct cell line. *J. Biol. Chem.* 269:7081-7089.

Shears, S.B. (1996) Inositol pentakis- and hexakisphosphate metabolism adds versatility to the actions of inositol polyphosphates. Novel effects on ion channels and protein traffic. In Subcellular biochemistry. Vol. 26. myo-Inositol phosphates, phosphainositides, and signal transduction. Eds., Biswas, B.B. and Biswas, S., Plenum Press, New York, pp.187-227.

Shimizu, T., Naruse, M., Takeda, M., Nakamura, M., Yoshitomi, K., Imai, M. (1992) Mechanism of PGE2-induced cell swelling in distal nephron segments. *Am. J. Physiol.* 263:F824-832.

Siddiqui, R.A. and Exton, J.H. (1992) Phospholipid base exchange activity in rat plasma membrane. Evidence for regulation by G-protein and P_{2Y}-purinergic receptor. *J. Biol. Chem.* 267:5755-5761.

Sigworth, F.J. (1980) The variance of sodium current fluctuations at the node of Ranvier. *J. Physiol.* 307:97-129.

Silver, R.B., Frindt, G., Windhager, E.E., and Palmer, L.G. (1993) Feedback regulation of Na channels in rat CCT. I. Effects of inhibition of Na pump. *Am. J. Physiol.* 264:F557-F564.

Singh, A.K., Afink, G.B., Venglarik, C.J., Wang, R., and Bridges, R.J. (1991) Colonic Cl^- channel blockade by 3 classes of compounds. *Am. J. Physiol.* 260:C51-C63.

Smith, A.E. (1995) Treatment of cystic fibrosis based on understanding CFTR. In Ion channel and genetic diseases. Eds., Dawson, D.C. and Frizzell, R.A., The Rockefeller University Press, New York, pp.139-147.

Solc, C.K. and Wine, J.J. (1991) Swelling-induced and depolarization-induced Cl^- channels in normal and cystic fibrosis epithelial cells. *Am. J. Physiol.* 261:C658-C674.

Soslau, G. and Parker, J. (1989) Modulation of platelet function by extracellular adenosine triphosphate. *Blood* 74:984-993.

Spranzi, E., Djeu, J.Y., Hoffman, S.L., Epling-Burnette, P.K., and Blanchard, D.K. (1993) Lysis of human leukemia cells by extracellular adenosine triphosphate: Mechanism and characterization of the adenosine triphosphate receptor. *Blood* 82:1578-1585.

Steinmeyer, K., Ortland, C. and Jentsch, T.J. (1991) Primary structure and functional expression of a developmentally regulated skeletal muscle chloride channel. *Nature* 354:301-304.

Steinmeyer, K., Schwappach, B., Bens, M., Vandewalle, A., and Jentsch, T.J. (1995) Cloning and functional expression of rat ClC-5, a chloride channel related to kidney disease. *J. Biol. Chem.* 270:31172-31177.

Strange, K. and Jackson, P.S. (1995) Swelling-activated organic osmolyte efflux: A new role for anion channels. *Kidney Int.* 48:994-1003.

Strohmeier, G.R., Reppert, S.M., Lencer, W.I., and Madara, J.L. (1995) The A_{2b} adenosine receptor mediates cAMP responses to adenosine receptor agonists in human intestinal epithelia. *J. Biol. Chem.* 270:2387-2394.

Stutts, M.J., Chinet, T.C., Mason, S.J., Fullton, J.M., Clarke, L.L., and Boucher, R.C. (1992) Regulation of Cl^- channels in normal and cystic fibrosis airway epithelial cells by extracellular ATP. *Proc. Natl. Acad. Sci. USA* 89:1621-1625.

Stutts, M.J., Fitz, G., Paradiso, A.M., and Boucher, R.C. (1994) Multiple modes of regulation of airway epithelial chloride secretion by extracellular ATP. *Am. J. Physiol.* 267:C1442-1451.

Szerlip, H., Palevsky, P., Cox, M., and Blazer-Yost, B. (1991) Relationship of the aldosterone-induced protein, GP70, to the conductive Na^+ channel. *J. Am. Soc. Nephrol.* 2:1108-1114.

Tabcharani, J.A., Chang, X.-B., Riordan, J.R., and Hanrahan, J.W. (1991) Phosphorylation-regulated Cl^- channel in CHO cells stably expressing the cystic fibrosis gene. *Nature* 352:628-631.

Tabcharani, J.A., Low, W., Elie, D., and Hanrahan, J.W. (1990) Low-conductance chloride channel activated by cAMP in the epithelial cell line T84. *FEBS Lett.* 270:157-164.

Tada, S., Okajima, F., Mitsui, Y., Kondo, Y., Ui, M. (1992) P_2 purinoceptor-mediated cyclic AMP accumulation in bovine vascular smooth muscle cells. *Eur. J. Pharmacol.* 227:25-31.

Tatham, P.E.R. and Lindau, M. (1990) ATP-induced pore-formation in the plasma membrane of rat peritoneal mast cells. *J. Gen. Physiol.* 95:459-476.

Thiemann, A., Gründer, S., Pusch, M., and Jentsch, T.J. (1992) A chloride channel widely expressed in epithelial and non-epithelial cells. *Nature* 356:57-60.

Tominaga, M., Tominage, T., Miwa, A., Okada, Y. (1995) Volume-sensitive chloride channel activity does not depend on endogenous P-glucoprotein. *J. Biol. Chem.* 270:27887-27893.

Travis, S.M., Carson, M.R., Ries, D.R., and Welsh, M.J. (1993) Interaction of nucleotides with membrane-associated cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* 268:15336-15339.

Traynor-Kaplan, A.E., Buranawuti, T., Vajanaphanich, M., and Barrett, K.E. (1994) Protein kinase C activity does not mediate the inhibitory effect of carbachol on chloride secretion by T84 cells. *Am. J. Physiol.* 267:C1224-C1230.

Tsui, L.-C., Buchwald, M., Barker, D., Braman, J.C., Knowlton, R., Schumn, J.W., Eiberg, H., Mohr, J., Kennedy, D., and Plavsic, N., Zsiga, M., Markiewicz, D., Akots, G., Brown, V., Helms, C., Gravius, T., Parker, C., Rediker, K., and Donis-Keller, H. (1985) Cystic fibrosis locus defined by a genetically linked polymorphic DNA marker. *Science* 230:1054-1057.

Turnheim, K., Costantin, J., Chan, S., and Schultz, S.G. (1989) Reconstitution of a calcium-activated potassium channel in basolateral membranes of rabbit colonocytes into planar lipid bilayers. *J. Membrane Biol.* 112:247-254.

Uchida, S., Sasaki, S., Furukawa, T., Hiraoka, M., Imai, T., Hirata, Y., and Marumo, F. (1993) Molecular cloning of a chloride channel that is regulated by dehydration and expressed predominantly in kidney medulla. *J. Biol. Chem.* 268:3821-3824.

Ussing, H.H. (1986) Epithelial cell volume regulation illustrated by experiments in frog skin. *Renal Physiol.* 9:38-46.

Vajanaphanich, M., Schultz, C., Rudolf, M.T., Wasserman, M., Enyedi, P., Craxton, A., Shears, S.B., Tsien, R.Y., Barrett, K.E., and Traynor-Kaplan, A. (1994) Long-term uncoupling of Cl^- secretion from intracellular calcium level by Ins(3,4,5,6)P_4. *Nature* 371:711-714.

Valeins, H., Merle, M., and Labouesse, J. (1992) Pre-steady state study of ß-adrenergic and purinergic receptor interaction in C6 cell membranes: Underlayed balance between positive and negative coupling to adenylyl cyclase. *Mol. Pharmacol.* 42:1033-1041.

Valera, S., Hussy, N., Evans, R.J., Adami, N., North, R.A., Suprenant, A., and Buell, G. (1994) A new class of ligand-gated ion channel defined by P_{2X} receptor for extracellular ATP. *Nature* 371:516-519.

Vallejo, M., Jackson, T., Lightman, S., and Hanley, M.R. (1987) Occurence and extracellular actions of inositol pentakis- and hexakisphosphate in mammalian brain. *Nature* 330:656-658.

Valverde, M.A., Díaz, M., Sepúlveda, F.V., Gill, D.R., Hyde, S.C., and Higgins, C.F. (1992) Volume-regulated chloride channel associated with the human multidrug-resistance P-plycoprotein. *Nature* 355:830-833.

Van Driessche, W. and Van Deynse, N. (1990) Analysis of transepithelial noise signals from ion channels: Advantages and limitations of the method. In Current topics in membranes and transport, Vol. 37. Channels and noise in epithelial tissues. Eds., Helman, S.I. and Van Driessche, W., Academic Press, San Diego, California, pp. 37-59.

van Slegtenhorst, M.A., Bassi, M.T., Borsani, G., Wapenaar, M.C., Ferrero, G.B., de Conciliis, L., Rugarli, E.I., Grillo, A., Franco, B., Zoghbi, H.Y., and Ballabio, A. (1994) A gene from Xp22.3 region shares homology with voltage-gated chloride channels. *Hum. Mol. Gen.* 3:547-552.

Vincent, P. (1992) Cationic channels sensitive to extracellular ATP in rat lacrimal cells. *J. Physiol.* 449:313-331.

Volk, K.A., Husted, R.F., Pruchno, C.J., and Stokes, J.B. (1994) Functional and molecular evidence for *Shaker*-like K^+ channels in rabit renal papillary epithelial cell line. *Am. J. Physiol.* 267:F671-678.

Volpe, P. and Alderson-Lang, B.H. (1990) Regulation of inositol 1,4,5-trisphosphate-induced Ca^{2+} release. II. Effect of cAMP-dependent protein kinase. *Am. J. Physiol.* 258:C1086-C1091.

Von Kugelgen, I. and Starke, K. (1991) Noradrenaline-ATP cotransmission in the sympathetic nervous system. *Trends Pharmacol. Sci.* 12:319-324.

Wainwright, B.J., Scambler, P.J., Schmidtke, J., Watson, E.A., Law, H.-Y., Farrall, M., Cooke, H.J., Elberg, H., and Williamson, R. (1985) Localization of cystic fibrosis locus to human chromosome 7cen-q22. *Nature* 318:384-385.

Wagner, J.A., Cozens, A.L., Schulman, H., Gruenert, D.C., Stryer, L., and Gardner, P. (1991) Activation of chloride channels in normal and cystic fibrosis airway epithelial cells by multifunctional calcium/calmodulin-dependent protein kinase. *Nature* 349:793-796.

Wang, W.-H. (1994) Two types of K^+ channel in thick ascending limb of rat kidney. *Am. J. Physiol.* F599-F605.

Warth, R. and Greger, R. (1993) The ion conductances of CFPAC-1 cells. *Cell Physiol. Biochem.* 3:2-16.

Watras, J., Bezprozvanny, I., and Ehrlich, B.E. (1991) Inositol 1,4,5-trisphosphate-gated channels in cerebellum: presence of multiple conductance states. *J. Neurosci.* 11:3239-3245.

Webb, T.E., Simon, J., Krishek, B.J., Bateson, A.N., Smart, T.G., King, B.F., Burnstock, G., and Barnard, E.A. (1993) Cloning and functional expression of a brain G-protein-coupled ATP receptor. *FEBS Lett.* 324:219-225.

Weiss, H. and Lang, F. (1992) Ion Channels activated by swelling of Madin Darby Canine Kidney (MDCK) cells. *J. Membrane Biol.* 126:109-114.

Welsh, M.J. (1987) Electrolyte transport by airway epithelia. *Physiol. Rev.* 67:1143-1184.

Welsh, M.J., Li, M., and McCann, J.D. (1989) Activation of normal and cystic fibrosis Cl^- channels by voltage, temperature, and trypsin. *J. Clin. Invest.* 84:2002-2007.

Welsh, M.J. and McCann, J.D. (1985) Intracellular calcium regulates basolateral potassium channels in a chloride-secreting epithelium. *Proc. Natl. Acad. Sci. USA.* 82:8823-8826.

White, R., Woodward, S., Leppert, M., O'Connell, P., Hoff, M., Herbst, J., Lalouel, J.M., Dean, M., and Van de Woude, G. (1985) A closely linked genetic marker for cystic fibrosis. *Nature* 318:382-384.

Wilk-Blaszczak, M.A., French, A.S, and Man, S.F.P. (1992) 5 pS anion channels in human airway epithelial cells. *Biomed. Res.* 13:143-148.

Worrell, R.T., Butt, A.G., Cliff, W.H., and Frizzell, R.A. (1989) A volume-sensitive chloride conductance in human colonic cell line T84. *Am. J. Physiol.* 256:C1111-C1119.

Worrell, R.T. and Frizzell, R.A. (1991) CaMKII mediates stimulation of chloride conductance by calcium in T84 cells. *Am. J. Physiol.* 260:C877-882.

Wu, J., Zhang, J.J., Koppel, H., Jacob, T.J.C. (1996) P-glycoprotein regulates a volume-activated chloride current in bovine non-pigmented ciliary epithelial cells. *J. Physiol.* 491:743-755.

Xie, W., Kaetzel, M.A., Bruzik, K.S., Dedman, J.R., Shears, S.B., and Nelson, D.J. (1996) Inositol 3,4,5,6-tetrakisphosphate inhibits the calmodulin-dependent protein kinase II-activated chloride conductance in T84 colonic epithelial cells. *J. Biol. Chem.* 271:14092-14097.

Yakel, J.L., Warren, R.A., Reppert, S.M., and North, R.A. (1993) Functional expression of adenosine A_{2b} receptor in Xenopus oocytes. *Mol. Pharmacol.* 43:277-280.

Yang, Y., Nunes, F.A., Berencsi, K., Furth, E.E., Gonczol, E., and Wilson, J.M. (1994) Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc. Natl. Acad. Sci. USA* 91:4407-4411.

Zabner, J., Couture, L.A., Smith, A.E., and Welsh, M.J. (1994) Correction of cAMP-stimulated fluid secretion in cystic fibrosis airway epithelia: efficiency of adenovirus-mediated gene transfer in vitro. *Hum. Gene. Ther.* 5:585-593.

Zhang, Y.X., Yamashita, H., Ohshita, T., Sawamoto, N., and Nakamura, S. (1995) ATP increases extracellular dopamine level through stimulation of P_{2Y} purinoceptors in rat striatum. *Brain Res.* 691:205-212.

Zhou, H., Tate, S.S., and Palmer, L.G. (1994) Primary structure and functional properties of an epithelial K channel. *Am. J. Physiol.* 266:C809-C824.

Zhou, Q.Y., Li, C., Olah, M.E., Johnson, R.A., Stiles, G.L., and Civelli, O. (1992) Molecular cloning and characterization of an adenosine receptor: the A3 adenosine receptor. *Proc. Natl. Acad. Sci. USA* 89:7432-7436.